

SPECIFIC REQUIREMENTS FOR THE PRODUCTION AND CONTROL OF LIVE AND INACTIVATED VACCINES INTENDED FOR FISH

Guideline Title	General Requirements for the Production and Control of Live and Inactivated Vaccines intended for Fish.
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Additional Notes	This note for guidance is intended to provide general guidance on the type of data which should be included in applications for marketing authorisations for bacterial and viral vaccines intended for administration to fish. It is intended to supplement Directive 81/852/EEC as amended, and should be read in conjunction with that Directive. Cross-references to the notes for guidance on <i>General Requirements for the Production and Control of Live Mammalian Bacterial and Viral Vaccines for Veterinary Use</i> and <i>General Requirements for the Production and Control of Inactivated Mammalian Bacterial and Viral Vaccines for Veterinary Use</i> are made.

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ANNEX

SPECIFIC REQUIREMENTS FOR THE PRODUCTION AND CONTROL OF LIVE AND INACTIVATED VACCINES INTENDED FOR FISH

This document is intended to provide guidance on the type of data which should be included in applications for marketing authorisations for viral and bacterial vaccines for fish. It is intended to supplement Directive 81/852/EEC as amended by Directive 92/18/EEC and must be read in conjunction with that Directive. A special attention should be given to the investigation into the possible spread of live vaccine strains to wild fish species.

The principle for the production and control of mammalian vaccines apply equally to fish vaccines. Where requirements have been included in the *General Requirements for the Production and Control of Live Mammalian Bacterial and Viral Vaccines for Veterinary Use* (GRLMV) or the *General Requirements for the Production and Control of Inactivated Mammalian Bacterial and Viral Vaccines for Veterinary Use* (GRIMV), these requirements have not been repeated here, but a cross-reference has been given.

I. GENERAL REQUIREMENTS FOR FISH VACCINES

All vaccines intended for use in fish shall normally comply with the GRIMV and GRLMV, as modified by this note for guidance.

Any material of piscine origin in the production and control of virus vaccines must originate from hatchery and fish farms of specified health status as defined in Annex A to this note.

II. LIVE VACCINES

PRODUCTION

1. STARTING MATERIAL

1.2 Cell substrates

1.2.1 General requirements

The general requirements of GRLMV apply.

1.2.2 Requirements for cell lines

If a virus can be grown efficaciously on cell cultures based on a seed lot system of established cell lines, no primary cells should be used.

Cell lines of piscine origin used for the production of vaccines for fish should be free from extraneous agents listed in the Annex to this note.

Cell lines of mammalian origin should comply with GRLMV section 1.2.

Cell substrates of avian origin should be derived from SPF flocks meeting the requirements specified in the General Monograph for Veterinary Vaccines in the European Pharmacopoeia.

1.2.3 Requirements for primary cells

The requirements for GRLMV 1.2.3 to 1.2.3.2 apply.

If a vaccine has to be produced on primary fish cells, the seed material should be obtained from a specific pathogen free farm with a complete protection from introduction of disease. The farm shall comply with the requirements in the annex.

1.3 Virus seed

The requirements of GRLMV 1.3 to 1.3.5 apply.

1.3.1 Extraneous agents

The test for extraneous agents specified in GRLMV 1.3.5 should be carried out as indicated in item 1.2.2 of this document.

1.4 Bacterial seed

The requirements of GRLMV 1.4 to 1.4.3 apply.

2. FINISHED PRODUCT – ASSAY RESULTS REQUIRED IN THE APPLICATION FOR MARKETING AUTHORISATION

All assays conducted with groups of fish should be duplicated or triplicate in order to take the tank effect into account.

2.1 Safety

The samples for the safety testing shall be taken from batches produced according to the manufacturing process described in the application for marketing authorisation.

The test shall employ the target species of fish. If a vaccine is intended for use in several species the test must be employed in each species.

The fish shall be observed and any morbidity/mortality recorded. After at least 14 days the remaining fish should be slaughtered and examined for signs of systemic and/or local reactions.

Any possible injection site reactions shall be addressed and suitable studies to reveal the occurrence or magnitude of such reactions should be documented.

The withdrawal period before slaughtering shall be documented.

2.2 Efficacy

Efficacy experiments shall be performed on a sufficient number of target species and fish. The following should be well documented: The efficacy of all the vaccine strains, the efficacy for all the species and age/size for which the vaccine is intended, e.g. oral, injection, dipping and the vaccination scheme recommended by the manufacturer, the temperature in the environments recommended for vaccinations.

If established, the nature and level of immune response following vaccination shall be documented. The duration of immunity, assessed by challenge, and the tolerance/side effects shall be documented in each case.

Field trials

Safety and efficacy must be studied in field trials performed on a sufficient number of target species distributed in more than one premises. The parameters mentioned for the experimental safety and efficacy studies should be looked for in the field trials and temperature in the environments should be recorded.

3. TEST ON THE FINISHED PRODUCT – BATCH TESTING

The requirements of GRLMV apply. Testing for safety and efficacy shall be carried out as follows:

3.1 Safety

The test shall employ the target species of fish. If a vaccine is intended for use in several species, the species most sensitive to infectious agents in question should be used.

For vaccines intended for individual administration, at least 30 susceptible fish should be vaccinated and for vaccines intended for oral-, spray-, bath- or dip-vaccination, at least 50 fish should be employed. Fish of minimum age and/or size recommended for vaccination should be used. Vaccination should be carried out according to label instructions. A control group of a composition similar to the vaccinated group should undergo mock-vaccination. During the following 21 days, vaccinates and controls should be held at an environmental temperature relevant to the species and observed every day for mortality and adverse effects. Mortality among vaccinates should not exceed that of controls, and no clinical symptoms of ill health should appear in any surviving vaccinates. The cause of mortality should be assessed. If in the case of 30 vaccinates, more than 2 fish die or if more than 3 out of 50 vaccinates die during the observation period, the safety test must be repeated once. If in the second test the specified limits for mortality are again exceeded, the batch is considered unsafe and must be discarded.

3.2 Potency

Where the viral or bacterial titre in the vaccine does not correlate with the protection and a potency test is required to comply with the requirements of GRLMV 3.7, the following test should be carried out.

The test shall employ the target species of fish. If a vaccine is intended for use in several species, the species most sensitive to the infectious agent in question should be used. Fish of the minimum age and /or size recommended for vaccination should be employed.

For each strain of organisms contained in the vaccine, at least 50 fish should be vaccinated according to label instructions.

Challenge should take place at least 28 days following vaccination. For each strain contained in the vaccine, one group of vaccinates and a simple group of mock-vaccinated, susceptible fish should be exposed to the corresponding virulent organism. The challenge should cause at least 70 per cent morbidity or mortality in control fish. Addition of virulent organisms to the water is the preferred method of exposure; however with some species of microorganisms, individual challenge, e.g. intra-muscular or intra-peritoneal

administration, may be necessary in order to obtain acceptable morbidity/mortality among controls.

For some diseases it may be acceptable to introduce the virulent organisms into the water by means of infected fish where vaccinates and controls are held in the same tank.

Following challenge, all groups shall be kept at an environmental temperature relevant to the species and must be observed each day until at least 21 days after challenge and until it is established that no further mortality is occurring.

For all strains of organisms contained in the vaccine, morbidity and mortality should be significantly lower in vaccinated fish than in mock-vaccinates.

III. INACTIVATED VACCINES

PRODUCTION

1. STARTING MATERIAL

1.2 Cell substrates

See requirements for live vaccines for fish.

1.3 Virus seed

See requirements for live vaccines for fish.

1.4 Bacterial seed

See requirements for live vaccines for fish.

2. FINISHED PRODUCT – ASSAY RESULTS REQUIRED IN THE APPLICATION FOR MARKETING AUTHORISATION

See requirements for live vaccines for fish.

3. TEST OF THE FINISHED PRODUCT – BATCH TESTING

See requirements for live vaccines for fish.

ANNEX

Health requirements for pisciculture establishments (fish farms) acting as suppliers of material of piscary origin used in the production and control of virus vaccines intended for fish.

The requirements are not exhaustive; they will be in accordance with the provisions of Directive 91/67/EEC and its amendments and adapted to progress in knowledge.

1. Fish farms acting as suppliers of material of piscary origin used for the production and control of virus vaccines intended for fish must maintain SPF status with respect to the following pathogens:

Viral agents

Viral Haemorrhagic Septicaemia Virus (VHSV) type I, II, III

Infectious Haematopoietic Necrosis Virus (IHNV)

Spring Viraemia of Carp Virus (SCVC)

Infectious Pancreatic Necrosis Virus (IPNV)

Protozoal agents

Myxosma cerebralis

Bacterial agents

Yersinia ruckeri

Vibrio anguillarum

Aeromonas salmonicinarum

2. In order to document continuous freedom from the pathogens mentioned in section 1, the establishment must be subjected to a disease control on a regular basis, including laboratory examination of suitable specimens collected on the premises.
3. In order to become eligible as supplier, the establishment must have been subjected to official disease control carried out on a regular basis for at least 2 years, during which period no evidence of pathogens on the premises must be revealed. In addition, it must be documented that natural conditions and preventative measures make it highly unlikely for contamination of the premises with the pathogens to occur, e.g. through the water supply or through wild or restocking fish.



COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

NOTE FOR GUIDANCE:

**ENVIRONMENTAL RISK ASSESSMENT FOR IMMUNOLOGICAL VETERINARY
MEDICINAL PRODUCTS**

ADOPTION BY CVMP	24 July 1996
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COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

ENVIRONMENTAL RISK ASSESSMENT FOR IMMUNOLOGICAL VETERINARY MEDICINAL PRODUCTS

I - BACKGROUND AND INTRODUCTION

This guidance concerns the environmental risk assessment needed to comply with the requirements of Part III E of Directive 81/852/EEC. This requires an assessment to be undertaken of the potential risk to the environment from use of the immunological veterinary medicinal product, before it may be placed on the market. This assessment must address the risks arising from each of the components of the product, not just the risk from live organisms in vaccines. The assessment need not, however, address the possible outcome of misuse or accidents.

The Directive describes the process of assessment as being in two phases. The first phase is compulsory and should indicate the potential exposure of the environment to the product and the level of risk associated with any such exposure.

Where it is concluded that the risk is low there will generally be no need to proceed to the second phase and no further investigations will be required.

In the majority of cases, the nature of Immunological Veterinary Medicinal Products is such that they will have a very low environmental risk potential. It can be expected that only in exceptional circumstances a Phase II assessment will be necessary.

The following guidance should be used for the Phase I assessment and to reach a conclusion on the risks. If further investigations and a Phase II assessment is required, the same assessment procedure and format should also be used.

The level of detail to be considered in a risk assessment will depend on circumstances. It will be lower, for example, where it is immediately obvious that the hazards and hence the consequent risks are low or that the proposed control measures are clearly adequate to limit the contact between the product and the environment. For example, for inactivated vaccines to be administered by injection, the hazards and risks from the active ingredients are likely to be negligible.

The risk assessment is intended to be an overall statement reflecting all the information contained in the dossier.

Although, wherever possible, the risk assessment should be based on quantifiable outcomes, it is recognised that many of the judgements must necessarily be qualitative.

How much information is needed on any particular point will depend on its importance in the assessment and the extent to which it is generally accepted material. Much of the information that is required should have already been addressed in the rest of the section on Safety, particularly with respect to live organisms in the product, through studies of excretion and spread and in the considerations of the risk to unvaccinated animals of the same or other species. There is no need to spell out in great detail what is elsewhere in the dossier or in text books or literature, but a clear cross reference to this information should be provided. In addition, within this section, sufficient information should be provided for the logic of the argument to be clear and enough justification should be included on any unusual or particularly important points for the assessment to be testable.

Note that it is always permissible for the applicant to assume the worst and act accordingly, if the cost of gathering the information (by experimentation or review) for a more precise assessment is disproportionate.

II - FRAMEWORK FOR RISK ASSESSMENT

The aim of the risk assessment is to identify hazards, to estimate the likelihood that the hazards will lead to actual harm and to take decisions regarding the appropriate control measures. The main elements of a risk assessment are therefore:

- i. hazard identification;
- ii. assessment of exposure to the hazard and the likelihood that the hazard will occur;
- iii. assessment of the consequences of that exposure;
- iv. a assessment of the level of risk (by consideration of the severity of any adverse consequences and the likelihood that they will occur);
- v. selection and assignment of appropriate control measures (risk management), as far as possible.

III- ASSESSMENT OF RISK

1. Hazard identification

In the context of this guidance, hazards are defined as those features of the substance which have the potential to cause harm to the environment, either directly (such as infection of a non-target-species by vaccine virus) or through some form of possible event (such as the infection from organisms excreted by the vaccinated animal). It is important to be exhaustive in the identification of possible hazards and not to discount at this stage any of the hazards given below on the basis that they are unlikely to occur. The assessment of possible exposure and likelihood are separate stages of the assessment process.

This stage of the assessment should aim to identify all possible factors contributing to adverse effects and should include the following:

1.a. Capacity of live organisms to transmit to non-target species

The specificity of the host range is very important for veterinary products. Any likely changes as a result of the attenuation should be taken into account.

1.b. Shedding of live product organisms (route, numbers, duration)

The extent to which the product organisms in live vaccines multiply in the host, can be excreted and spread will have been studied as part of the safety studies. Many products may well consist of attenuated or replication defective organisms and the likelihood of exposure may be less than that associated with the wild type organisms. However, the potential for organisms passaged from animal to animal to become less attenuated and more likely to be excreted and spread must be taken into consideration.

1.c. Capacity to survive, establish and disseminate

This is also a key consideration: if an organism is not capable of surviving, then other hazards are likely to be minimised. The risk assessment associated with live vaccinal organisms could be completed at this stage if the risks to the environment are low or effectively zero. However, if it is likely that the organism could survive for a sufficiently long period for it to cause harm, and possibly establish and disseminate in the environment, then not only this hazard but also other hazardous characteristics need to be considered.

1.d. Pathogenicity to other organisms

The pathogenic properties of many organisms are well documented; these should be identified, if appropriate. Consider whether a change in host range could occur as a result of the attenuation which has been undertaken.

1.e. Potential for other effects of live product organisms

Consider whether the organism might have the potential to exert other effects such as the transmission and replication of viruses in other organisms as a result of the effects of recombination.

Consider whether the organisms have the capacity to transmit potentially harmful characteristics to other organisms e.g. via plasmids.

1.f. Toxic effects of the product components

The potential of each of the components in the product to exert a toxic effect must be considered. In the case of products which are administered by injection, no detailed assessment of the potential risk of the excipients is likely to be required when the substances are of biological origin or form part of the animal's normal diet. For other pharmacologically active excipients, some information may be available from the data gathered or generated in support of the application in accordance with Council Regulation (EEC) No. 2377/90.

In the case of products administered other than by injection, the assessment should include assessment of the total quantity of each substance that is released into the environment.

1.g. Toxic effects of excreted metabolites

Consider whether excreted metabolites of the product components administered to the target species could present a hazard.

In the case of products which are administered by injection, no detailed assessment of the potential risk of the excipients is likely to be required when the substances are of biological origin or form part of the animal's normal diet. For other pharmacologically active excipients, some information may be available from the data gathered or generated in support of the application in accordance with Council Regulation (EEC) No. 2377/90.

2. Assessment of likelihood

The next step is to estimate the likelihood (probability and frequency) of hazard(s) being manifested. A key factor in determining this is the potential receiving environment. This includes the wider as well as the local environment in which the product is intended or likely to be used.

Particular characteristics of the local environment that could contribute to manifestation of the hazard should be identified and assessed. Climatic, geographical and soil conditions, demographic considerations, the types of fauna and flora in the potential receiving environment are some of the important ones.

Consideration should be given to any potential exposure of the environment to the product and the magnitude and duration of such exposure.

When estimating probabilities and frequencies for live vaccinal organisms, consideration should include the number of organisms that might reach the environment since the probability that a

hazard will be realised will often be influenced by the number of viable organisms in the environment. This could be due, for example, to excretion, as well as being influenced by the number of organisms per dose and whether many doses will be given at one time, in mass vaccination, or a single or small number of doses will usually be given.

For toxic components in the product and hazardous metabolites, the quantities and concentrations of these that might reach the environment should be considered.

The following should be taken into account:-

(i) Type of packaging and procedures before and after administration

The packaging should allow any initial preparatory steps (e.g. reconstituting freeze-dried preparations) to be undertaken in a safe and aseptic manner. However the proposed method of preparation and administration will have a bearing on the degree of exposure of the environment to the product and need to be considered. For example, single dose preparations for administration to a companion animal in the surgery is likely to result in less exposure than mass medication of farm animals including poultry and fish. It may be appropriate to consider who is likely to administer the product (veterinary surgeon or farmer) and the likelihood of any necessary instructions for safe use of products being achievable. It will also be necessary to consider whether or not unused product can be readily disposed of in a reliably safe manner.

(ii) Route of administration (parenteral vs. oral vs. ocular vs. spray)

It may be expected that there is more opportunity for exposure to the product when the product is administered by spray, orally or ocularly than by injection. Oral, bath or dip vaccination of fish may result in considerable exposure.

(iii) Shedding of live product organisms (route, numbers, duration)

See II 1.b.

For the hazard 'survival capacity' of living organisms, it is appropriate to assess the proportion of the organisms that are likely to survive. If the organism has pathogenic characteristics, assess the proportion of target species in the environment likely to be affected, including taking into consideration, the likelihood of the organism to spread to or reach these species.

Similar considerations need to be applied to all toxic components of the product and hazardous metabolites eg. will the chemical preservative reach susceptible non-target animal species or susceptible plants.

It is recommended that the possibility of exposure and the likelihood of hazards occurring is expressed as 'high', 'medium', 'low' or negligible', although it is recognised that this requires subjective judgement.

3. Assessment of the consequence of a hazard occurring

For each hazard of the product identified, whenever it is possible or probable that the components in the product will reach the environment, it must be considered whether that environment would cause or allow the hazard to be realised. Thus, again, the characteristics of the potential receiving environment need to be considered.

For vaccinal organisms or excreted passaged organisms, the main consideration will be the likely presence or absence of susceptible non-target species in the potentially affected environment.

An assessment of the magnitude of harm is based on the assumption that the hazard will be realised. Inevitably there will be a degree of judgement in making the assessment, but the consequences should be described as 'severe', 'medium', 'low', or 'negligible'. A 'severe' consequence might be a major change in the numbers of one or more species leading to negative effects on the functioning of the ecosystem and/or other connected ecosystems. It is unlikely that the changes would be reversible. A 'low' consequence might be if any change in population densities is such that it has no negative effects on ecosystem function and no impact on endangered or beneficial animal or plant species.

The above illustrations reflect the potential effect of the product on populations. In some cases, however, it may be more appropriate to consider the likely effects on individual organisms, for example endangered mammals. In most cases it should be possible to use the guidelines to assess in qualitative terms the degree of harm which a particular product might cause.

4. Assessment of level of risk

Having identified any hazards and assessed the degree and likelihood of exposure and the consequences of that exposure it is necessary to evaluate the risk associated with each hazard. Risk is generally held to be the product of exposure/likelihood and consequence. It is inevitably always going to be difficult to 'multiply' qualitative statements such as 'high' and 'low', but the table in Annex 1 should help this process. The risk matrix is not definitive and there will always be some scope for flexible, case by case evaluation. In many cases, it will be necessary to decide between one of two outcomes and as in the earlier parts of the process, some justification for the choice should be provided. In addition, a range of risks may be apparent if more than one hazard is being evaluated. There will therefore be a need to make an overall assessment of the risk taking all factors into consideration.

Once an overall assessment of the risk associated with each hazard has been produced it will be necessary to evaluate the significance of the risk.

5. Selection and assignment of appropriate control measures (risk management)

If the environmental risks are not as low as reasonably practicable, the process of risk assessment in relation to that hazard should be repeated to ascertain whether the application of additional management techniques could reduce the level of risk. Consideration might be given, for example, to limiting the proposed routes of administration to those likely to lead to a lower

level of risk. If it is considered that there is insufficient knowledge to come to a satisfactory conclusion, further studies and a phase II assessment should be undertaken.

IV - FORMAT FOR PRESENTATION OF CONCLUSIONS OF RISK ASSESSMENT

Applicants may find the following structure useful to record their risk assessment.

1. Summary

Summary of the overall risk of damage to the environment from the proposed marketing of the product.

2. Assessment of the risks to the environment

2.a. Hazard identification Hazardous characteristics of the product that could, in certain circumstances, lead to harm to the environment

2.b. Assessment of likelihood

2.c. Assessment of the consequence

2.d. Assessment of level of risk

2.e. Assessment of the overall risk to the environment (the total risk after consideration of the risk of each of the hazards occurring): High, medium, low, effectively zero.

ANNEX I

ESTIMATION OF RISK

Consequence of Hazard	Likelihood of Hazard Occurring			
	High	Moderate	Low	Negligible
Severe	High	High	Medium	Effectively Zero
Medium	High	High	Medium/Low	Effectively Zero
Low	Medium/Low	Low	Low	Effectively Zero
Negligible	Effectively Zero	Effectively Zero	Effectively Zero	Effectively Zero

This matrix is not intended to be definitive, but illustrative of the way in which an estimate of risk might be obtained from the consequence and likelihood that a hazard will be realised. Different components may be differently weighted, however, depending on the knowledge and experience of the product.



COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

NOTE FOR GUIDANCE:
REQUIREMENTS FOR COMBINED VETERINARY VACCINES

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1. INTRODUCTION

1.1 Scope

There is a strong demand for harmonised regulations and a common approach to combined vaccines in the European Union. This note for guidance gives advice to manufacturers and regulatory authorities to ensure that combined vaccines as defined in this note for guidance fulfil the standards of quality, safety and efficacy. It will enable the competent authorities to arrive at their decisions by reference to uniform criteria for combined vaccines and will therefore avoid differences in evaluation.

This guideline does not address requirements for individual specific combined vaccines. For products with components which are the subject of relevant European Pharmacopoeia monographs, some specific guidance is provided in those monographs. In the case of combined vaccines, for each component that is the subject of a monograph in the European Pharmacopoeia, the provisions of that monograph apply to that component modified where necessary as indicated in the following paragraphs and as indicated in the European Pharmacopoeia monograph “Vaccines for Veterinary Use”.

The rules governing veterinary medicinal products in the European Union are naturally applicable to combined vaccines to ensure their quality, safety and efficacy. The aim of this guideline is to ensure harmonisation of the requirements for the registration of combined vaccines in the European Union.

This guideline does not deal with simultaneous administration of separate vaccines.

1.2. Definitions

Combined (multivalent) vaccines are immunological products intended for

- i) immunisation against different infectious diseases; or
- ii) immunisation against multifactorial infectious diseases caused by different species, types or variants of pathogens
- iii) combinations of (i), (ii)

In technical terms the following products are considered as combined vaccines and their use should be specifically addressed in the Summary of Product Characteristics:

- a) products supplied in a single primary container;
- b) products supplied in several primary packages and packed in a common secondary package with instructions for admixture before use and administration in a single site

1.3 Points to Consider for Combined Vaccine Research and Development

The development of combined vaccines is not straightforward. Each combination should be developed and studied individually in terms of quality, safety and efficacy. Initially, this includes the pharmaceutical development to establish the correct formulation, the stability of and compatibility between the individual components in the combined vaccine, including preservatives, excipients and stabilisers, inactivating agents and adjuvants.

In combined vaccines, the presence of more than one component can often cause an interaction, leading to either a diminished or an increased response to individual components, compared to when the specific component(s) is administered alone. Under justified circumstances, the diminished immune response to individual components can be acceptable.

Such interactions are often immunological in nature, but may also be caused by other factors with less direct effects on the immune system.

The potential for a vaccine combination to be more reactogenic than the individual components has to be considered. The effect of increasing toxoid load from conjugates needs to be considered. The possibility of increasing endotoxin content, particularly where components derived from Gram negative bacteria are used, also needs to be taken into account. This could be a limiting factor, for example, in combinations of inactivated bacterial vaccines containing *E. coli*.

The physical and chemical compatibility of these components deserves carefully drafted formulation studies preceding any clinical trials. Possible deleterious effects of vaccine components on each of the active constituents of both viral and bacterial products should be controlled. With an increasing number of components to be incorporated into a combination the problem of the volume to be administered arises. In this context, studies to concentrate the antigen amount in a given vaccine may have to be performed.

When an adjuvant is used to augment the immune response to a combined vaccine, special problems may appear. For adsorbed vaccines adjuvanticity is usually dependant on each vaccine component being firmly bound to the adjuvant, and on the presence of non-occupied sites on the adjuvant. The adsorption procedure to be used in routine manufacture must be clearly established during development since changes can result in variation in the antigen-adjuvant binding and a lack of batch to batch consistency.

2. QUALITY ASPECTS

2.1 Manufacturing and control requirements

The requirements for manufacture and control of combined vaccines are given in relevant guidelines for assuring the quality of veterinary biological products. For many combined vaccines there are already requirements available for the individual components. The methods of preparation vary according to the type of vaccine, as described in specific European Pharmacopoeia monographs, EU-legislation and manufacturers' production and control records. These methods are designed to guarantee the quality, safety and efficacy of each vaccine component, with greatest emphasis on maintaining the appropriate antigenic properties and acceptable safety as well as to ensure freedom from contamination with extraneous agents.

2.2 Formulation

Specific requirements for the ingredients such as adjuvants, antimicrobial agents, inactivating agents, antibiotics used in production, preservatives present in the finished product are specified in various guidelines for production and control of immunological veterinary medicinal products (Volume VII of the rules governing medicinal products in the EU) and in various monographs of the European Pharmacopoeia.

2.3 Stability

Stability testing of biological products requires that primary data to support a requested storage period should be based on long-term, real-time, real-condition stability studies. In the case of combined vaccines there are some special points to be considered: stability data are requested for the combined vaccine as finished product. The shelf life begins on the date of initiation of the last valid batch potency test of the first component tested and the maximum length of the storage period should always be based upon the duration of satisfactory batch potency of the least stable component and the shortest shelf life proven.

2.4 Batch testing

The following tests must be performed on the finished combined product as defined under point 1.2.

2.4.1. Safety testing

Two doses of an inactivated vaccine or ten doses of a live vaccine, *if EP monographs do not require other dosages*, are administered by a recommended route. If in cases of combined vaccines containing a live active substance the volume of 10 doses of vaccine could be unacceptably large, the number of doses of the finished product used in the test may be reduced.

If the safety test is performed on the finished combined product, no test with single components is necessary.

2.4.2 Potency testing

The aim of the release testing of a given vaccine batch is to show that this batch is consistent with and equivalent to the successive batches and to the batches that have been shown to be efficacious in clinical trials. See also position paper on batch potency testing of immunological veterinary medicinal products (CVMP/IWP/038/97-FINAL).

The testing of individual primary packages is acceptable.

3. SAFETY ASPECTS

The safety of the combined product shall be demonstrated with the vaccine containing the largest number of components. Safety tests carried out on the combined vaccines may be regarded as sufficient to demonstrate the safety of the individual components or vaccines containing a smaller number of components providing the components (antigens, composition of excipients and/or adjuvants) are identical in each case and it is only the number of active ingredients which is changed.

3.1 Laboratory trials

The safety testing requirements of Directive 81/852/EEC must be addressed during the development testing of the product. The batches used in the tests on the finished products should contain the maximum titre or potency of each component. The combined product must be shown to be safe in single, overdose and repeated dose safety studies (Annex II, III C 1,2,3) and in any studies of the effect on reproduction, immunological function and interactions (Annex II, III C 4,5,8). Where there is a component, which may pose a particular risk for an adverse effect on the immune system, or the reproductive performance it may be appropriate also to

conduct studies on the individual component. If the agents cannot be distinguished and re-isolated separately, each related group of live organisms may be tested separately to address the requirements for live vaccines (Annex II, III C 6).

3.2 Field trials

The test results of a combined vaccine of a larger combination may be used as results for combined vaccine of a smaller combination providing the components (antigens, composition of excipients and/or adjuvants) are identical in each case and it is only the number of active ingredients which is changed.

See also under Point 4.

4. EFFICACY ASPECTS

Combination vaccines should be studied for their appropriate efficacy parameters in the target species. Normally, the efficacy of each of the components of combined products shall be demonstrated using the combined vaccine. Test results from large combinations should be acceptable for smaller combinations of the same antigens and, under conditions specified below, vice versa, providing the components (antigens, composition of excipients and/or adjuvants) are identical in each case and it is only the number of active ingredients which is changed. Possible synergistic interactions should be evaluated.

When appropriate, dose response studies for different batches of the combined vaccine should show very similar results, indicating a consistency in the product and in the resulting immune response. Repeated challenges can be avoided by measuring parameters correlated to the immune response or which have been demonstrated to be good indicators to avoid repeated challenges.

4.1 Laboratory trials

Protection should be demonstrated by challenge with toxin or a virulent strain(s) of each organism against which the vaccine is intended to protect.

The deletion of challenges is only acceptable in rare cases and must be fully justified.

The batches used in the tests should contain the minimum titre or potency of ~~each~~ the component *to be tested in the study*.

In order to avoid repeated challenges, the results from challenge studies with monovalent vaccines or lesser combinations, which have received a marketing authorisation *in accordance with EU requirements* may be used as efficacy data for a larger combination *and vice versa*, providing certain conditions apply. Firstly, the components (antigens, composition of excipients and/or adjuvants) must be identical in each case and it is only the number of active ingredients, which is changed. There must be a marker for protection for the particular active ingredient(s), (e.g. a serological response has been shown to provide evidence of protection). Data must be provided from serological studies to show that the compounds in the larger combination provide a serological response at least equal to that stimulated by the same components in the smaller combination or superior to a threshold of acceptability. Trials must be conducted in a way that is capable of detecting any significant difference in the responses to the different products.

4.2 Field trials

The test results of a combined vaccine of a larger combination may be used as results for combined vaccine of a smaller combination *and vice versa* providing certain conditions apply. The antigen(s), which are present in the larger combination but not present in the smaller combination, should have no interference effect. There should be evidence of bio-equivalence e.g. data that the antibodies measured provide clear evidence of protection, together with data from serological studies to show that the components in the smaller combination provide a serological response which is at least equal to that stimulated by the same components in the larger combination.



The European Agency for the Evaluation of Medicinal Products

EMA/CVMP/682/99 - FINAL

COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

NOTE FOR GUIDANCE:

DURATION OF PROTECTION ACHIEVED BY VETERINARY VACCINES

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DURATION OF PROTECTION ACHIEVED BY VETERINARY VACCINES

1. Introduction.

Directive 81/852/EEC states that all claims on the efficacy of the vaccines, including the duration of protection, and the regimens of administration contained in the application for a marketing authorisation shall be fully supported by the data from specific laboratory trials and field studies.

The European Pharmacopoeia states that any claim regarding the duration of protection achieved by vaccines shall be supported by the data from trials, without specifying the methods and requirements concerned.

The formulation of claims shall take into account the guidance contained in the Position paper on Indications and Specific Claims for immunological veterinary medicinal products.

In the case of vaccines, efficacy means induction of immunity to provide protection. The nature, degree, onset and duration are the main parameters of the protection.

The duration of protection achieved by vaccines is influenced by a number of factors such as the characteristics of the causal agent(s) of the disease, the epizootiology of the infection, the immunogenicity of the active substances of the vaccines and the nature of the immune-response of the target animals.

The duration of protection may be different for each category of vaccines and for the products within a category of vaccines, as a consequence of the quality and properties of the products concerned.

In addition, it has to be realised that the duration of protection achieved under field conditions can vary from time to time and can also vary from that achievable under laboratory conditions because of the influence of a number of factors, such as the field conditions of use, e.g. exposure to the infectious agent(s) and the health, condition and immunological status of the animals to be vaccinated.

The guidance in this paper is specifically aimed at vaccines against infectious diseases, although the same principles may be applicable to products which through immunological mechanisms affect the physiological functions of an animal.

In order to avoid frequent vaccinations, it is recommended to study the vaccines in a manner which demonstrate the actual duration of protection provided and to develop products that provide as long a duration of protection as possible.

2. Scope of the guidance.

The scope of this guidance is to define what data shall be generated from trials and how such data can then be used to support claims for the duration of protection achieved by veterinary vaccines.

3. Definitions.

For the purpose of this guidance the following definitions apply:

Basic vaccination scheme:

One or more administrations of a vaccine with the second and any recommended subsequent doses given a short time after the first dose. This is the vaccination scheme which is necessary to obtain and maintain the level of protection claimed by the applicant.

Re-vaccination scheme:

One or more administrations of a vaccine used to maintain its initial protective effects, induced by the basic vaccination scheme. The first (or only) dose of the re-vaccination is given a relatively long time (e.g. 3 months or more, depending on the species and the disease) after the basic vaccination scheme.

Regimen of vaccination:

The basic vaccination scheme and re-vaccination scheme altogether.

4. Duration of Protection

For most infectious animal diseases, one administration of a vaccine does not provide protection which will last for the natural or economical life of the animals. Therefore, regimens of vaccination are in most cases necessary.

Where there are more administrations of vaccine recommended in the basic vaccination scheme and/or the re-vaccination scheme, the protection during the interval(s) between the administrations has to be also addressed.

Where there is no recommendation for more than one administration of a vaccine or for only a basic vaccination, this implies in principle a life-long protection thereafter. As the natural or economical duration of the life of animals differs for species and categories within a species and regionally, the claimed duration of protection shall then be specified and supported by sufficient data.

In cases of seasonal diseases, it will be sufficient to demonstrate the duration of protection in the year after vaccination until the end of the natural occurrence of the disease. The protection in the seasonal period(s) in the next year(s), with or without re-vaccination, will have also to be addressed.

It is not possible to generalise about the minimum period for which a vaccine shall be expected to provide protection. However, in all cases, the duration of protection demonstrated shall be justified in relation to the length of time for which an animal is likely to be at risk.

5. Data Requirements

The duration of protection that can be claimed is the longest interval between the administration of a vaccine to target animals and the observed protection against the required challenge.

The studies required to generate this data shall be conducted under well-controlled conditions. If the necessary studies are very difficult to conduct in laboratory conditions, field trials only may be carried out. Since the duration of protection from vaccination is being measured in the studies, it shall be ensured that the vaccinated target animals are not exposed to intercurrent field infection which could boost the immunity. It is usually necessary, therefore, to maintain unvaccinated target animals in contact to act as sentinels in laboratory or field studies to provide the necessary assurances on this point.

The results from vaccination-challenge trials conducted under laboratory conditions shall be, unless justified, supplemented with sufficient data from well-controlled field studies. In these field studies, target animals are vaccinated in the field and undergo thereafter a natural challenge in the field or an experimental challenge under laboratory conditions.

A. Duration of protection from the basic vaccination scheme.

a. For active immunity.

The duration of protection provided by the basic vaccination scheme shall usually be demonstrated by a challenge of vaccinated animals just before the recommended time for the start of the re-vaccination scheme.

b. For passive immunity.

The duration of protection of the progeny from passively acquired antibodies shall usually be demonstrated by a challenge at the time of natural susceptibility of the offspring of females which have been vaccinated at the maximum interval recommended between the basic vaccination scheme and parturition or lay.

In addition, data shall be presented to support the duration that is claimed for the protection of the offspring.

The nature of the disease concerned, including the age of the animals at which the onset of the disease usually occurs, as well as the onset of age resistance needs to be taken into account.

B. Duration of protection from the re-vaccination scheme.

The re-vaccination shall result in a protection that is quantitatively and qualitatively at least equivalent to the response to the basic vaccination scheme. This is best demonstrated by challenge trials at suitable times between the end of the scheme and the end of the claimed period of protection thereafter.

Vaccination-challenge trials, in particular those to study the duration of protection, are expensive and time-consuming as well as having animal welfare issues involved. In order to limit the need for frequent challenges in studies on the duration of protection, it may be considered:

- to challenge of a more limited number of immunised animals
- to measure the protection using a suitable indicator other than challenge.

Antibodies against the causal agent(s) of the infectious disease may be an example of such indicators.

For such an indicator to be acceptable, evidence shall be provided to show that the indicator plays a substantial role in the protection of the target species and that there is a sufficient qualitative and quantitative relationship between the indicator and the protection of the target species against the disease concerned.

It must be demonstrated (via serological studies or other markers of protection) that the level of response before revaccination or at the end of the protection period can be considered as equal to the one observed at the time of challenge used to demonstrate the efficacy.

The data generated by a combined vaccine can be used to support the protection to be induced by a vaccine containing fewer active ingredients provided these products are manufactured according to the same process, have the same composition (with the exception of the additional antigens) and there is no evidence of a negative or positive interference from the other active ingredients present in the combined vaccine.

As the immune systems of even related, species are different, the use of data from trials in a species other than the target species will normally not be acceptable.

In view of the diversities of species and diseases, each case has to be considered on an individual basis.

In the case of fish vaccines, long term studies are often difficult to perform under laboratory conditions. Therefore, it is essential that appropriately designed field trials are carried out.



COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS

NOTE FOR GUIDANCE

FIELD TRIALS WITH VETERINARY VACCINES

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FIELD TRIALS WITH VETERINARY VACCINES

1. Introduction

The efficacy and safety of veterinary vaccines shall in the first instance normally be demonstrated by experiments under laboratory conditions.

Council Directive 81/852/EEC and the European Pharmacopoeia state that, unless justified, the results from laboratory trials shall be supplemented with data from field trials. It is also stated that, when efficacy cannot be demonstrated by laboratory trials, field efficacy trials alone may be acceptable.

There are two main reasons for carrying out field trials with veterinary vaccines:

- a. The verification that the results of the efficacy and safety trials, under field conditions and on a large scale, reflect those observed in the laboratory trials with the target animals.
- b. The investigation of efficacy items that cannot be studied sufficiently well under laboratory conditions in the target animals. Examples of such items are:
 - Diseases where a suitable experimental infection model does not exist.
 - Certain diseases caused by more than one causal agent.
 - Cases where special husbandry facilities are involved (e.g. drinking water vaccines for poultry, water qualities and temperatures for fish vaccines).
 - Certain diseases where environmental factors play a major role in the aetiology.

Deviations from these guidelines may be acceptable provided they are scientifically justified. The guidance should be followed for products intended for use in food-producing and companion animals.

2. Scope of the guidance

The scope of this guidance is to advise on how to perform field trials with veterinary vaccines, what criteria shall be taken into account, what data are expected and how the data shall be analysed.

This document covers in particular field efficacy trials and, where relevant, safety trials.

3. Definition

For the purpose of this guidance the following definitions apply:

Field trial: A scientific investigation of a veterinary vaccine under field conditions and in target animals (in terms of animal species and categories), using the product as recommended.

Comparator Product: A product that has been authorised in accordance with the EU requirements with similar indications and recommendations for use and used accordingly.

4. Implementation of field trials

Directive 81/852/EEC and EU guideline "Good clinical practice for the conduct of clinical trials on veterinary medicinal products in the European Union" give the basic standards for the conduct of field trials with veterinary vaccines.

Such trials shall be well planned, controlled and monitored as well as carried out in representative animal houses/husbandry practices and geographical regions. The trials shall follow a study plan made beforehand.

Field trials shall at least cover the major uses. The vaccines shall be administered by the most critical route (e.g. intranasal), method (e.g. spray) and regimen of vaccination to be recommended in the most relevant category of target species. It may not be necessary to include all recommendations, provided that sufficient data from laboratory trials are supplied to cover all uses of the vaccine not included in field trials.

4.1. Field efficacy trials

Normally one dose of vaccine used shall not contain significantly more than the minimum titre of the vaccine agent(s) or batch potency(ies) to be stated on the label and, for live vaccines, the vaccine agent(s) shall be at the highest attenuated passage level that will be present in a batch of the vaccine. Provided that sufficient data have been presented for all efficacy aspects from laboratory trials using batches of vaccines with minimum titre or batch potency, the use of a batch of vaccine with an intermediate titre or batch potency shown to be representative of those found in routine production of the vaccine is acceptable.

Whenever possible, the field trial shall include the challenge of vaccinated animals by exposure to natural infection. However, it is recognised that a natural infection can neither be predicted nor standardised. It may not appear at the appropriate time and may be too weak or too low in incidence or, in the case of multivalent vaccine testing, not all natural challenges may occur in the study timeframe. Intercurrent infections with the same or other complicating pathogens may also occur.

4.1.1. Parameters

The parameters to be measured shall be clearly defined in the study protocol and justified in relation to the indications and specific claims for the vaccine.

Conversely, justification shall be given for not measuring parameters that are usually related to the disease concerned.

Two types of parameters exist: the main parameters (e.g. mortality, morbidity, lesions, weight gain, epizootiological impact) and the indicators (e.g. serological response).

For an indicator to be acceptable as a correlate of vaccine efficacy, it shall be shown that a sufficient qualitative and quantitative correlation exists between the indicator measured and the claimed protection in the target species.

If relevant and available, test methods shall be employed that can differentiate naturally infected from vaccinated animals.

4.1.2. Controls and trial design

The trial shall, unless justified, compare a group of vaccinated animals with an equivalent group of unvaccinated or placebo controls.

Where vaccination of whole herds is proposed the need for this shall be justified. In such cases, comparison with animals vaccinated with a comparator product may be used when available. For modified live vaccines, whose vaccine agent(s) spread, it is necessary to separate vaccinates from controls. In such cases separate housing of these both groups is justified.

The choice of the controls shall be justified. It is necessary to define in the study protocol what purpose the control group serves. This shall include:

- Evidence that exposure to infection took place.
- A group of animals against which the vaccinated animals can be compared in a valid manner.

For such a comparison to be valid:

- The controls and vaccinated animals shall be as contemporaneous as possible, preferably investigated at the same time;
- The animals of both groups have to be randomised according to the experimental unit;^a
- The environment in which the two groups of animals are housed shall be as equivalent as possible (i.e. same farm/ barn/batch) or at least as similar as possible (e.g. same farm/different barn/same batch);

^a An experimental unit has to be defined in the protocol and is the smallest number of animals in a defined environment on which a statistical analysis can be based.

- The challenge infection shall be as similar as possible in the two groups of animals. This will not be the case if cohorts consist of exclusively vaccinated animals or controls. In this case, repetition of the trials under the same conditions is necessary, using truly randomised groups. The rearing of both groups together may affect the infection rate.

The use of historical data for control purposes is rarely acceptable but where they are used they shall have been shown to be consistent over a representative length of time and well documented.

When investigating a combined vaccine, the control group may comprise animals vaccinated with a product formulated to contain all the components of the vaccine except the component under study.

Ideally, the trials shall be double blind, placebo controlled, but this is often difficult to realise in practice. The need for placebo controls depends on the study plan. If the parameter to be measured is a subjective one (e.g. coughing), then the trial must be done in a blind manner and either placebo controls shall be included or the person who measures this parameter shall have no information on the details of the vaccination.

It is recognised that in some circumstances (e.g. enzootic diseases) inclusion of controls may be difficult. However, even when this is not possible, sufficient evidence shall be presented that the vaccine is having a demonstrable beneficial effect.

4.1.3. Comparator product

The comparator product and the vaccine under study should claim the same indication.

When the vaccine under study is being compared with a comparator product, a group of controls shall still be included whenever possible. Even if this is not possible, sufficient evidence shall be presented that both products are having a demonstrable beneficial effect rather than just comparing the results of the two groups of animals.

4.1.4. Exposure to infection

Clear evidence that the vaccinated animals and controls have been exposed to the concerned pathogen shall be given. In principle, the level and moment of exposure shall be the same in both groups of animals. Observation of signs of disease is rarely sufficient by itself and clinical records shall be supported by laboratory tests. In principle, the agent(s) itself shall be detected and identified. In the case of live vaccines, the isolated field strains shall, whenever possible, be differentiated from the vaccine strains. Serology, performed on a statistically sufficient number of animals, may be a supportive measure to demonstrate the exposure to infection. Care must be taken as this may be open to different interpretation. The serological method(s) used shall be validated and the same as used in the laboratory trials.

The causes of any deaths or unexpected signs of disease related to the parameters being measured shall be determined, unless justified. In avian industrial production, standard procedures for diagnosis should be used to determine the cause of death.

If justified, some of the vaccinated animals may undergo an experimental challenge under laboratory conditions, but shall be shown not to have been naturally infected beforehand.

4.1.5. Intercurrent infections

Infections with intercurrent agents other than those under study that may influence the parameters being measured, might affect the outcome of the trial. Such an influence on the trial can be reduced considerably if the vaccinated and control animals are investigated contemporaneously and allocation of both groups of animals has been made at random.

4.1.6. Pre-existing antibodies

Pre-existing antibodies against the agent(s) of the vaccine may be:

- Maternally derived.
- Due to infection.
- Due to vaccination.

If the indication or specific claims for the vaccine are related to efficacy in the presence of maternal antibodies against the vaccine agent (s), the trial protocol shall include animals with titres of these antibodies normally occurring in the field.

Where pre-existing antibodies due to previous exposure to the concerned or related agents are present, the trial can still be acceptable if the immunological status of the vaccinated animals and controls at the time of vaccination is known and a justification for their use is given.

In all cases, field trials shall not be carried out in animals that have been vaccinated with products containing the same active substances as the vaccine under study.

4.2. Field safety trials

For field safety trials, one dose of vaccine shall not contain significantly less than the maximum titre of the vaccine agent(s) or batch potency to be stated on the label and, for live vaccines, the vaccine agent(s) shall be at the lowest attenuated passage level that will be present in a batch of the vaccine.

The field safety trials are, in the first instance, to verify the safety of the vaccine under field conditions after one administration of one dose of vaccine as well as after repeated administration (s) depending on the recommendations.

Part of the data on the safety of the vaccine may be generated from the field efficacy trials.

4.2.1. Parameters

Field safety trials shall be designed to detect both local and systemic reactions to vaccination.

Field trials are also used to investigate the possible side-effects of vaccination with the product in relation to special items, if relevant. Examples of such systemic effects include allergic reactions, mortality, anorexia, pyrexia, changes in behaviour, weight gain, feed conversion, carcass quality, milk/wool/fur production, egg production and hatchability of breeding eggs and male and female fertility. In the case of live vaccines, the behaviour of the vaccine agent(s) in animal populations should be documented. In terms of local reactions, the size, duration and nature of any lesions appearing at the sites of injection shall be monitored and recorded.

4.2.2. Controls and trial design.

The trial shall normally compare a group of vaccinated animals with an equivalent group of unvaccinated or placebo controls.

The choice of the controls shall be justified. The control group shall comprise animals against which the vaccinated animals can be compared in a valid manner.

See further, where relevant, paragraph 4.1.2.

4.3. Animal welfare Considerations

Field trials can have implications of importance for animal welfare. The inclusion of control animals may lead to an increase in the prevalence of the disease . However, it is important to recognise that the use of controls holds the promise of preventing or reducing the future incidence of the disease through the release of efficacious vaccines. Furthermore, carrying out field trials in animals under normal husbandry conditions may reduce the number of animals needed for laboratory trials.

Special care should therefore be taken with the study plan in order to respect animal welfare when carrying out such studies.

5. Analysis and interpretation

According to Council Directive 81/852/EEC all available data of field trials shall be included in the dossier of the Application for a Marketing Authorisation. Only data of valid field trials may support such an Application and in particular all relevant details should be given of any incomplete or abandoned test or trial.

The analysis of the data of field efficacy trials shall be related to the Indication and specific claims made for the vaccine and the parameters measured (see Position paper on indications and specific claims for immunological veterinary products).

The analysis of the data of field safety trials shall be related to the recommendations for the administration of the vaccine.

Careful consideration shall be especially given to:

- The study plan.
- The plan for analysis.
- The evaluation of the data.
- The statistical evaluation, including confidence limits, of the data.
- The hypothesis for risk of errors.
- The randomisation of the various groups of animals.
- The number of animals required, including eventual losses during the trial.

In the case of efficacy as judged by serology, the titres measured in vaccinated animals shall be not significantly lower in the target animals used in the field trials than those in the laboratory trials. In the case of a marker vaccine, special attention should be paid to properties of the marker.

6. Deviations from the basic principle

In cases of vaccines against notifiable and /or exotic animal diseases for which vaccination is not allowed in the European Union, it may be difficult to find other suitable areas to carry out the required field trials. In such cases the need for extensive laboratory trials may be increased. Such cases are judged on an individual basis to determine if there is a zoo-sanitary legal requirement to restrict the efficacy and safety investigations to laboratory trials. Data from field trials conducted outside the EU, especially if conducted according to Good Clinical Practice, may be considered in support of applications for such vaccines.

When the vaccine is intended against an animal disease that occurs only rarely and sporadically in the field and where a suitable laboratory test model exists, it may be acceptable under certain conditions to limit the requirement for field efficacy trials. Such cases shall also be judged on an individual basis. In such cases the need for extensive laboratory trials may also be increased.

In cases where the vaccine is intended for use in minor species, a limitation of the field trial requirements may be considered. Justification shall be given and the decision made on an individual basis.

In the circumstance where a side-effect occurs very rarely (e.g. allergic reactions) extensive post marketing surveillance rather than extended field trials may be considered for the purpose of gaining a Marketing Authorisation for the vaccine.



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

14 November 2011
EMA/CVMP/IWP/314550/2010
Committee for Medicinal Products for Veterinary Use (CVMP)

Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines

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This guideline replaces the following guidance document:

Specific requirements for the production and control of live and inactivated vaccines intended for fish
7Blm9a



Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines

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Executive summary

This document provides information on items to be considered in the design and conduct of studies to support the safety and efficacy of immunological veterinary medicinal products (IVMPs) in finfish.

The guideline outlines important items to take into account for both laboratory scale size and field trials so that the studies are representative of the safety and efficacy of the vaccine when administered in accordance with its intended use (e.g. type of fish to be used; water conditions, method of administration, use of control groups etc).

The guideline also outlines aspects to be considered in the determination of the duration of immunity for vaccines intended for use in fish.

1. Introduction (background)

This document provides guidance in respect of the design of studies for the evaluation of the safety and efficacy of immunological veterinary medicinal products (IVMPs) for use in finfish. The procedures outlined should be considered for all submissions, but may not be applicable for all IVMPs for use in aquaculture. If certain aspects are modified or omitted, justification should be provided.

In principle the results of all studies should be applicable irrespective of where they are carried out; however, the applicant should take into account the various conditions (e.g. climatic, disease situation, water temperature and salinity) as these may influence the outcome of the studies.

For some fish species / diseases reductions in the requirements may be acceptable as outlined in EMA/CVMP/IWP/123243/2006: 'Guideline on data requirements for IVMPs intended for minor use or minor species/limited markets.'

Guidance on quality requirements for fish vaccines is outlined in the general guideline: 'Requirements for the production and control of immunological veterinary medicinal products.'

2. Scope

The aim of this guideline is to provide guidance regarding the conduct of studies to demonstrate the target animal safety and efficacy for immunological veterinary medicinal products intended for use in farmed finfish.

3. Legal basis

This document should be read in conjunction with Directive 2001/82/EC as amended and in particular Annex I thereof, Directive 2010/63/EC (regarding the protection of animals used for experimental and other scientific purposes) and relevant European Pharmacopeia (Ph. Eur.) monographs (e.g. Ph. Eur. 0062, 5.2.6 and 5.2.7).

4. General considerations for studies involving fish

The following animal welfare concerns should be taken into consideration when designing studies to test the safety and efficacy of IVMPs in fish:

Personnel conducting the studies should be appropriately trained to detect behavioural changes / signs of illness in the participating fish.

The method used to identify vaccinated and controls fish should involve the least harmful technique.

The number of fish in the vaccinated and control groups should be sufficient to obtain statistically significant and clinically reliable results. However, for vaccination-challenge studies, the possibility of reducing the number of control non-vaccinated fish should be investigated as these fish will suffer disease and associated distress.

Mortality as an evaluation parameter in vaccination-challenge studies should be questioned whenever possible; humane endpoints are preferable. Moribund fish should be humanely slaughtered.

Relevant items to be considered in the design / performance of both safety and efficacy studies for fish vaccines are outlined below.

4.1. *Fish to be used*

The fish to be used must not have been vaccinated against any of the antigens in the vaccine and should be from a population shown to be free from specific antibodies against any of the vaccine antigens against which protection is claimed.

The range of species, ages, sizes, weights and physiological status (e.g. smoltification, sexual maturation) of fish used in the studies must be representative of those to which the vaccine will be administered in the field for commercial purposes. For laboratory studies, fish of the minimum recommended vaccination age or size should be used.

The origin/varying genetics of the experimental fish used is important to obtain valid reproducible results. Variation from those which will be encountered under commercial use conditions should be addressed. Fish used in studies should preferably be derived from stock which will be used commercially.

All finfish species shall be identified by their colloquial name followed in parenthesis by the Latin or Linnean description.

The allocation of fish to vaccinated and control groups should be done randomly, using an appropriate method. The numbers of fish per group should be justified and the sample size in each group should be sufficient to allow the results to be statistically significant and clinically reliable.

If vaccinated and control fish are not housed in the same tank, to overcome tank effects which may be experienced between groups of fish which are kept under identical conditions but in different tanks, measures should be taken to overcome such effects e.g. a minimum of two tanks could be used for each of the vaccinated and control groups. Effects of stocking density on the parameters to be determined should be taken into consideration.

4.2. *Water conditions*

Water quality parameters such as temperature and salinity (e.g. freshwater versus seawater) used in each laboratory study during vaccination should be relevant to the environment under which the vaccine will be used for commercial purposes. For challenge model development consideration can also

be given to possible changes in the water conditions/environment which fish may be exposed to during the life cycle (e.g. changes associated with transfer to sea etc).

Water quality parameters such as temperature and salinity should be documented in each study report.

The different climatic conditions and water temperatures within the Community should be considered, when relevant for the fish species/disease in question. Some studies may need to be performed both at high and low temperature for the relevant fish species/disease distribution. Similarly, some studies may need to be performed following the main production practices for the species (e.g. S0 and S1 stocking for salmon).

The chosen conditions should be justified by the applicant for each study.

To account for the fish being poikilothermic animals and taking into account the fact that immunity in fish is temperature dependant and that the frequency and intensity of injection site reactions increases with higher water temperatures, comparative data from safety and efficacy laboratory studies involving fish should be based on "degree-days".

4.3. Vaccine to be administered

The vaccine formulation to be administered in the safety and efficacy studies should be the final formulation proposed for marketing. Data from studies performed with a formulation(s) which differs to the proposed final commercial formulation can only be used as supportive information unless the applicant can justify that the differences have no impact on the safety or efficacy profile of the vaccine.

4.4. The vaccine dose (i.e. dose volume and amount) and administration method(s) employed in the safety and efficacy studies must represent those proposed for commercial use of the vaccine. Study reports

The applicant is encouraged to standardise study protocols and study reports as far as possible to facilitate the comparison of study results and the possible extrapolation between species.

Each laboratory study or field study and the conditions under which they are performed should be described in detail.

Separate reports on all studies, whether favourable or not, should be provided. All adverse events should be reported. An explanation of non-specific mortalities and comments on any physical or behavioural abnormalities should be provided.

5. Laboratory studies

Requirements for laboratory scale safety and efficacy studies for veterinary vaccines are outlined in Directive 2001/82/EC as amended, and in particular Annex I thereof and relevant Ph. Eur. monographs (e.g. Ph. Eur. 0062, 5.2.6 and 5.2.7).

Additional items to be considered in the design and performance of laboratory safety and efficacy studies for fish other than those referred to in the above mentioned documents are outlined in the following sections of this guideline.

5.1. Safety studies

The safety should be determined for all the proposed target species, unless otherwise justified.

Studies performed in one species of fish may be considered relevant for the evaluation of safety in a second species of fish, provided that the recommended conditions for use of the vaccine in both species are similar e.g. similar fish size/water temperature and quality etc at the time of vaccination. In such a case there should be supportive data from studies in the second species. It may for example be considered unnecessary to carry out laboratory safety studies in trout if such studies have been carried out on other species (e.g. salmon), and if field studies in trout are available.

The use of a negative control group may be useful in the evaluation of the safety of certain vaccines. For example, for parenteral vaccines inclusion of a mock-vaccinated group (e.g. saline) in the study should be considered to distinguish between reactions attributable to the vaccine itself and reactions associated with the injection process e.g. the anaesthetic used during vaccination and other manipulations.

In all tests, the vaccine and control should be administered in the same manner and control fish should be handled identically to vaccinated fish.

Details of the type of control group used should be clearly documented and the applicant should justify the contents of the control “vaccine”.

To assess the acute safety characteristics of the vaccine, the fish should be monitored daily for mortality/morbidity over a minimum of a 14 day period (or as recommended in the relevant Ph. Eur. monograph for specific vaccines) taking into account the optimal water temperature for the target species. At the end of the monitoring period, when appropriate the fish should be slaughtered humanely using a method described by Directive 2010/63/EC and examined for systemic and/or local reactions macroscopically and/or microscopically as appropriate.

For parenteral vaccines, post mortem examination should include investigation of the occurrence of effects such as pigmentation (e.g. melanisation) and adhesions. (e.g. measured using the Speilberg score – refer to Annex 1 of this guideline for details).

The nature and frequency of all adverse reactions should be monitored and recorded.

It is important to take into account the possible adverse effects of vaccine administration on development over the life span of the target fish species. This is particularly important in the case of parenteral vaccines as adhesions may have a negative effect on spawning, and adhesions/pigmentation may result in rejection or down-grading of fish at slaughter.

On this basis, the safety studies should be capable of allowing a prediction to be made of the safety profile over the average life span of the fish species. For example weight gain over the life span (for food producing fish) and for parenteral vaccines the percentage of fish down-graded on quality grounds due to adhesions/pigmentation at slaughter time etc are important aspects to be considered.

It may be more appropriate to evaluate the long term safety effects of vaccine administration over the life span in field studies as discussed in Section 6 below.

5.2. Efficacy studies

The efficacy studies should be designed to support the use of the recommended vaccine dose and administration schedule (including the recommended re-vaccination, if applicable) in providing optimum protection against the claimed indications.

The parameters evaluated should be appropriate to the proposed indications (including onset and duration of immunity claims (if relevant to the study design)) and should be pre-determined prior to conducting the studies.

Every study should be designed to allow for appropriate statistical evaluation. A sample size analysis should be presented. The statistical evaluation methods should be justified.

The challenge model used in each study must be justified by the investigator and the relevance to the natural disease situation should be discussed. Items to be considered include: the relevance of the challenge organism(s) to the disease(s) against which protection is claimed, the method of administration of the challenge organism (e.g. cohabitant, injection etc), the water temperature, the timings of (i) the challenge and (ii) the recording of the evaluation parameters. In the case of claims for protection against mortality, it is important that the evaluation period is of sufficient duration to reveal the total development of the mortality curve, both in control and vaccinated animals as vaccination may delay the onset of mortality. In efficacy studies where mortality is expected, the use of validated humane endpoints should be considered.

Data are required to support each proposed indication for all target species for which efficacy is claimed. Where justified, challenge studies can be replaced by an alternative method based on antibody response when a suitable correlation with efficacy has been demonstrated.

The studies should include a control group and the applicant should justify the choice of control group (mock-vaccinated or non-vaccinated) used.

Data from well controlled laboratory studies are preferred wherever relevant models are available, and field studies should serve to confirm the findings from the controlled studies. However, it is recognized that for some disease situations in fish, no or only poor challenge models exist. In such situations, with appropriate justification, more emphasis may be placed on field studies conducted under conditions which reflect the disease situation in the field as discussed in Section 6 below.

6. Field studies

The scope of the field studies is to ensure that the vaccine is efficacious and safe in the diversified conditions for aquaculture found in Member States for the relevant fish species. The field studies are to be performed in established commercial farms. Controlled semi-field studies performed in large scale research facilities may also be relevant, if justified. A satisfactory number of sites with conditions representative for the normal in-use conditions should be used. The applicant should justify the number of sites. The field studies should be performed in accordance with GCP as far as possible.

The number and suitability of the sites selected for the field studies should be justified by the applicant. These should be geographically well distributed to optimise the possibility of diversified environmental conditions, disease situation and management practices. Each site should have several pens or tanks with fish of the relevant size/age and physiological condition (e.g. smoltification, sexual maturation) for the proposed use of the vaccine. At least two of the pens or tanks, and preferably several pairs of pens/tanks should be used in the study per vaccinated and control group. The farmer should preferably be experienced in keeping detailed records on all important factors concerning the farm and its fish. Records on the source of fish and the disease history in different pens or tanks must be kept. Previous medication, use of chemicals and vaccines should be known. Daily records of outbreaks of disease, mortality and medication are required, as well as known and stable management practice concerning for example hygiene, feeding, handling and use of feed additives and chemicals.

Both a vaccinated and control group should be used. The allocation of the groups should be done randomly, using an appropriate method. The prevalence of disease, daily mortality, clinical symptoms and other relevant parameters should be comparable in the vaccinated and control group. The type of control group used (i.e. mock-vaccinated, non-vaccinated or positive control) should be justified. If a positive control (e.g. a comparator vaccine) is used, consideration should be given to maintaining a (small) group of non vaccinated fish in a separate test pen to serve as indicators of exposure to

disease(s) at farm level. Once the relevant infection has been diagnosed in the controls they can be slaughtered humanely using a method described by Directive 2010/63/EC.

For field studies involving multivalent vaccines where one or more new antigens have been added to a previously approved vaccine, the control vaccine should ideally contain the same antigens as the test vaccine with the exception of the new antigen(s).

Field studies in commercial fish farms should preferably be performed in farms known to be subject to spontaneous outbreaks of the disease(s) against which protection is claimed. Studies should thus be conducted at the time of year and under conditions relevant to the occurrence of a “natural challenge”. The method of identification and confirmation of the presence of the causal agent(s) for the natural challenge in each group is an important factor for field studies involving fish. The method used must be relevant to the disease situation and should be recorded for a representative number of fish in each group. Justification should be provided for the diagnostic method(s) and representative number of fish used with consideration being given to guidance on diagnostic methods from official disease control laboratories.

Information from studies where a natural challenge was not detected should be provided along with a discussion of the relevance of the parameters chosen as endpoints for the proposed claims. A full evaluation of the safety data from these studies should be provided.

Normally, data from both laboratory and full scale field studies will be required. Where appropriate the applicant should justify the lack of relevant data.

Omission of field studies and submission of laboratory studies only may be accepted if adequately justified by the investigator. For example, in case of a second species closely related to a first species for which the product is fully documented and where recognized models to establish vaccine efficacy (e.g. challenge or antibody response) exist, laboratory studies may be sufficient to document efficacy also in the second species.

In situations where field studies are not expected to be of value for assessment of efficacy due to absence / low occurrence of natural challenge and efficacy data are only available from laboratory studies, consideration should be given to including additional efficacy endpoints in the laboratory studies, and/or waterborne challenge in addition to challenge via injection in order to mimic the field conditions. For injection vaccines to be used for fish intended for human consumption and that are not covered by Guideline EMA/CVMP/IWP/123243/2006 (MUMS products), safety data from field studies (weight gain, local reactions) which are predictive of the safety over the life cycle, should however always be available.

7. Duration of immunity (DOI) claims

It is important that DOI claims are supported by reliable data. The following aspects should be considered:

Studies conducted under semi-field conditions where groups of fish are taken from the holding tank / cage / pen etc at different intervals and subjected to challenge infection or evaluation of a specific antibody response (where a suitable correlation with efficacy has been established) are useful in evaluating the DOI. For these studies, the relevance of the holding conditions used (e.g. freshwater vs seawater; water temperature / quality etc) to the conditions which will be encountered when the vaccine is used naturally in the field should be taken into account when proposing a DOI for the vaccine.

Field studies used to determine DOI should involve monitoring for the occurrence of disease / causal agent(s) at the participating site(s) on a regular basis to ensure that the disease is detected as soon as

possible after the outbreak. Large time periods between monitoring points could result in detection at a much later time than the actual outbreak of the disease. In addition, it is possible that natural exposure to the pathogen/s may boost immunity.

Ideally, the DOI claims will be based on a combination of the results from challenge studies conducted under semi-field conditions and disease monitoring data and serological testing results from the field studies.

In general for the DOI claims proposed for the SPC it is not necessary to refer to the design and the conditions used in the studies unless for specified reasons (e.g. different production methods exist for the target species, DOI has not been determined for all target species or different DOI for different target species).

If a reliable DOI cannot be determine from the challenge / field studies, this should be stated in the SPC.

Definitions

For the purpose of this guideline, the following definitions apply:

Finfish: A term used to separate true fish from shellfish, crayfish, jellyfish etc. All the species of fish mentioned in this guideline are examples of true finfish.

Degree days: The amount of degree days is determined by multiplying the water temperature each day with number of days. For example, 10 days with 5° C equal 50 degree days.

Parenteral administration: the vaccine is administered by injection.

Immersion administration: vaccine is administered by dipping or bathing the fish in an immersion bath/tank. Spray vaccination is a form of immersion vaccination.

Oral administration: vaccine is administered via the feed.

Annex 1

Speilberg scoring system (Midtlyng *et al.* 1996).

Score	Visual appearance of abdominal cavity	Severity of lesion
0	No visual lesions	None
1	Very slight adhesions most frequently localised close to the injection site. Unlikely to be noticed by laymen during evisceration	No or minor opacity of peritoneum after evisceration
2	Minor adhesions, which may connect colon, spleen or caudal pyloric caeca to the abdominal wall. May be noticed by laymen during evisceration.	Only opacity of peritoneum remaining after manually disconnecting the adhesions.
3	Moderate adhesions including more cranial parts of the abdominal cavity, partly involving pyloric caeca, the liver or ventricle, connecting them to the abdominal wall. May be noticed by laymen during evisceration.	Minor visible lesions after evisceration, which may be removed manually.
4	Major adhesions with granuloma, extensively interconnecting internal organs, which thereby appear as one unit. Likely to be noticed by laymen during evisceration.	Moderate lesions, which may be hard to remove manually.
5	Extensive lesions affecting nearly every internal organ in the abdominal cavity. In large areas, the peritoneum is thickened and opaque, and the fillet may carry focal, prominent and/or heavily pigmented lesions or granulomas.	Leaving visible damage to the carcass after evisceration and removal of lesions.
6	Even more pronounced than 5, often with considerable amounts of melanin. Viscera cannot be removed without damage to fillet integrity.	Leaving major damage to the carcass.



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

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Committee for Veterinary Medicinal Products (CVMP)

Guideline on data requirements for removing the target animal batch safety test for immunological veterinary medicinal products in the EU

Draft Agreed by Immunologicals working party	5 October 2011
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Guideline on data requirements for removing the target animal batch safety test for immunological veterinary medicinal products in the EU

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Executive summary

This guideline outlines the data requirements to be submitted by the marketing authorisation holder in order to waive the target animal batch safety test for the release of a batch of this product onto the market.

1. Introduction (background)

The European Pharmacopeia (Ph. Eur.) General monograph, Vaccines for Veterinary Use (0062), was revised in January 2004 (Supplement 4.6, Ph. Eur., 4th Edition) to state that for an established vaccine the routine application of the safety test may be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency if the competent authority considers that the changes introduced could adversely affect product safety. The requirements laid out in this guideline apply equally to both well established products and those that have been recently authorised. In the light of the changes to the Ph. Eur. monograph, the CVMP has developed this guideline for harmonising the data requirements for removing the target species safety test. The ability to waive this requirement relies, at least in part, on the assurance of product safety that is given by the current EU requirement for pre-authorisation safety tests (single dose, overdose, repeat dose). This guideline will need to be reviewed should these requirements be amended in the Ph. Eur. or as a result of international harmonisation.

2. Definitions

Final Batch

A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk vaccine, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final batch. Where a final bulk vaccine is filled and/or freeze-dried on several separate sessions, there results a related set of final batches that are usually identified by the use of a common part in the distinctive number or code; these related final batches are sometimes referred to as sub-batches, sub-lots or filling lots.

Final bulk vaccine

Material that has undergone all the steps of production except for the final filling. It consists of one or more monovalent pooled harvests, from cultures of one or more species or types of micro-organism, after clarification, dilution or addition of any adjuvant or other auxiliary substance. It is treated to ensure its homogeneity and is used for filling the containers of one or more final lots (batches).

Batch safety test

A safety test carried out on every batch of product to demonstrate the safety of the batch in the target species for which the product is intended. Where several batches are prepared from the same final bulk, the safety test is carried out on the first batch and then omitted for further batches prepared from the same final bulk.

Using at least the recommended route of administration posing the greatest risk, each batch of vaccine shall be shown to be safe in at least two susceptible animals from the most sensitive target species for which the vaccine is recommended.

3. Points to consider for removing the batch test for target animal safety

In general it is sufficient to evaluate existing information which is available from routine batch quality control and pharmacovigilance data, without the need for any additional supplementary studies. The data which should be presented to support such an application are presented below. However, this should not be taken as an exhaustive list, and should in all cases be accompanied by a summary report which brings together all of the data presented in terms of an overall assessment of the risk that waiving the requirement will represent.

3.1. *The characteristics of the product and its manufacture*

Directive 91/412/EEC covers the principles and guidelines of Good Manufacturing Practice (GMP) and provides assurance that products placed on the market place have been manufactured in a consistent and suitable manner. However, because of the inherent variability of biological products, there is a requirement for final product testing to provide further assurance that the product has been manufactured to the appropriate specifications. The target animal batch safety test on final product provides some assurance that the product will be safe in the target animal species even when administered at an overdose using the route of administration most likely to demonstrate a safety concern.

If the batch safety test is to be deleted as a final product test, a summary report should be provided which would encompass the inherent variability of manufacture of the product, the intrinsic safety margin and the validation which was undertaken to provide the necessary assurance that the product would always be manufactured to an acceptable level of quality and safety.

This would include the characteristics of the product, taking account of whether it has live and/or inactivated viral and/or bacterial components and for live components any residual virulence has been shown to be safe. The type of excipients, adjuvants and preservatives incorporated in the final formulation should be considered.

The applicant should also take account of the range of specifications for the manufacture of the product and how the extremes of the ranges and variability of the final formulation may influence the safety profile of the final product. In exceptional circumstances, where the safety threshold of the product is narrow, the applicant should justify that any issues related to batches at the limits of these parameters could be detected in the absence of the batch safety test. An example of this would be to examine the target ranges of endotoxin, which may be permitted in the final formulation, and how slight alterations in production conditions may influence the subsequent levels of endotoxin and the impact this may have on the safety profile of the resulting formulation.

For those circumstances when *in vivo* batch tests are conducted in the target species for reasons other than the target animal safety test, it is recommended that manufacturers use these tests to gain data of the safety of the vaccine in the target species.

3.2. Information available on the current batch safety test

Batch data should be submitted on at least 10 consecutive batches from separate final bulks, unless justified. These data may be submitted in the form of a table that lists the results for all finished product tests to demonstrate that the batches consistently met the agreed specifications and also details the antigen bulks used. The applicant should examine the variability of the local and systemic reactions observed in the batch safety test results and the nature of these reactions in relation to those observed in any developmental studies submitted in support of the marketing authorisation. The conduct of the trial shall be in accordance with the Ph. Eur. requirements in operation at the time when the tests were performed. There should be a thorough examination of any batches that have failed the batch safety test and this information, along with an explanation as to the reasons for failure, should be submitted to the regulatory authorities. Information on the number of years the product has been marketed in the EU should be provided.

3.3. Pharmacovigilance data

A satisfactory pharmacovigilance system should have been in place over the period during which the batches for which data are submitted were on the market.

Pharmacovigilance data to support the removal of the batch safety test should be provided. Marketing authorisation holders should follow the Note for Guidance for veterinary pharmacovigilance for marketing authorisation holders. The most recent Periodic Safety Update Report (PSUR) for the product should be submitted in the variation application to support the removal of the batch safety test. The summary report should include an overall assessment of the product, which would include taking account of the number of batches manufactured, the number of years the product has been on the market, the number of doses sold and the frequency and seriousness of any reactions relating to the safety in the target species and any investigations into the likely causes of these events.

3.4. Other data

Applicants should summarise the variation applications that have been submitted during the life of the marketing authorisation and any effects these changes may have had on the quality and safety profile of the product in the different categories of animals to which the product is given.

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Committee for Medicinal Products for Veterinary Use (CVMP)

Guideline on requirements for the production and control of immunological veterinary medicinal products

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This guideline replaces the following guidance documents:

General requirements for the production and control of live mammalian bacterial and viral vaccines for veterinary use 7BIm1a

General requirements for the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use 7BIm2a

Specific requirements for the production and control of avian live and inactivated viral and bacterial vaccines 7BIm3a

Specific requirements for the production and control of bovine live and inactivated viral and bacterial vaccines 7BIm4a

Specific requirements for the production and control of pig live and inactivated viral and bacterial vaccines 7BIm5a

Specific requirements for the production and control of ovine and caprine live and inactivated viral and bacterial vaccines 7BIm6a

Specific requirements for the production and control of equine live and inactivated viral and bacterial vaccines 7BIm7a

Specific requirements for the production and control of live and inactivated vaccines intended for fish 7BIm9a

Specific requirements for the production and control of immunosera and colostrum substitutes 7BIm12a

Specific requirements for the production and control of live and inactivated vaccines for cats and dogs 7BIm13a

Note for guidance Inclusion of antimicrobial preservatives in immunological veterinary medicinal products 7BIm14a

Public statement on the number of tests required to control for complete inactivation in inactivated vaccines (EMA/CVMP/IWP/596708/2010)

Guideline on requirements for the production and control of immunological veterinary medicinal products

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Executive summary

This document provides information on items to be considered for the production and control of all immunological veterinary medicinal products (IVMPs).

The guideline outlines important items related to the quality, safety and efficacy parts of the marketing authorisation dossier that are not sufficiently defined in the requirements of Annex I of Directive 2001/82/EC and the European Pharmacopoeia (Ph. Eur.). Therefore compliance with this guideline (and the above mentioned regulatory documents) provides an assurance that the IVMP will be considered satisfactory by all the Member States.

I Introduction

The guideline is intended to supplement Directive 2001/82/EC, the European Pharmacopoeia (Ph. Eur.), in particular Ph. Eur. monograph 0062 Vaccines for Veterinary Use, and relevant VICH guidelines. This guideline intends to clarify the requirements that are not covered by these. Principles of GMP are covered by specific guidance and by Directive 91/412/EC and are out of the scope of this guideline but they should be kept in mind in order to understand the rationale behind the requirements of this guideline.

All IVMPs shall normally comply with this guideline.

Compliance with the guidelines provides an assurance that the research and development work undertaken will be considered valid by all Member States. Nevertheless, in order not to place undue constraints on scientific research, an alternative approach to the one described in a guideline may be used, if it can be shown that this is justified.

Reductions in the requirements that may be acceptable are provided in a specific guideline "Guideline on data requirements for immunological veterinary medicinal products intended for minor use or minor species/limited markets".

Specific requirements for the production and control of immunosera and colostrum substitutes are attached as Annex 1 to this guideline.

Guidance on safety and efficacy requirements in the application for marketing authorisation for fish vaccines is outlined in "Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines".

II Quality

1. Devices

1.1. Definition

Annex I of Directive 2001/82/EC, Title II, Part 2.A, 1. Qualitative particulars states that:

"These particulars shall be supplemented ..., together with details of devices with which the IVMP will be used or administered and which will be delivered with the medicinal product. If the device is not delivered together with the IVMP, relevant information about the device shall be provided, where necessary for the assessment of the product."

For the purpose of this guideline, devices are defined as equipment used for the proper administration of IVMPs and which may influence the safety and efficacy of the product (e.g. devices for spray, intranasal, eye drop, intracutaneous, intrafollicular, *in ovo* administration).

1.2. Data requirements

As the use of a device can have an impact on the safety and efficacy of the IVMP, all the necessary data should be provided:

- A precise description of the device including an analysis of the possible influence on safety and efficacy of the IVMP.
- A detailed description of the sterilisation or disinfection of the device.
- A detailed description of the handling of the device.
- A clear statement of whether the device is delivered together with the IVMP or not
- A clear indication of the sources accessible in each Member state if the device is not delivered with the immunological veterinary medicinal product.

To avoid the use of inappropriate devices not evaluated in the safety and efficacy trials, the product information should indicate the type of device that should be used when administering the IVMP, and describe the physical and biological prerequisites and specifications of the device (e.g. volume of the delivered dose, pattern of distribution in skin, location of administration (intracutaneous, subcutaneous, and intradermal), pressure of the device, droplet size, etc.).

2. Starting materials and control during the manufacturing process

2.1. Absence of extraneous agents

When the Directive 2001/82/EC and the Ph. Eur. refer to the testing of potential contaminants, the table of extraneous agents (Volume 7, 7BIm10a and annex in 7BIm9a) should be taken into account.

2.2. Antibiotics

Antibiotics used during the production of an IVMP should be used under the restrictions of the Ph Eur monograph 0062 Vaccines for Veterinary Use.

Antibiotics used in the production of IVMPs may be present in the finished product. It is therefore recommended that for IVMPs intended for food producing species, antibiotics for which MRLs have been established in the relevant species should be used (ie the antibiotics should be listed in table 1 of the annex to Regulation 37/2010 for the relevant species). If an antibiotic not listed in table 1 of the annex to Regulation 37/2010 is used, then the applicant should address the consumer safety implications arising from its potential presence in the finished product. Applicants should note that residues of antibiotics not included in table 1 of Regulation 37/2010, found at residue control, would be considered as violative residue findings.

The number of antibiotics used has to be justified. The maximum concentration level of antibiotics used during the production should be defined. The level of remaining antibiotic content in the finished product should be indicated in the dossier and can be based on calculation.

2.3. Preservatives

In selecting a preservative system the applicant should consider

- the effectiveness against potential microbial contaminants;
- possible interaction with the formulation or container (for example, thiomersal is ineffective in sera, and can bind to SH groups and polymeric material);
- the potential pharmacological and toxicological effects on the target animal species, at the dose rates appropriate to the veterinary medicinal product;
- any maximum residue limits which have been fixed for the preservative substance(s), if appropriate;
- possible effects on testing of the immunological veterinary medicinal product, for example tests on cell cultures or mammalian species.

Long term experience with the use of the preservative in numerous similar products (e.g. thiomersal, formaldehyde) can be regarded as sufficient justification. The test procedures and microorganisms employed for demonstrating preservative efficacy should be as outlined in the Ph. Eur. monograph 5.1.3. Efficacy of Antimicrobial Preservation. The range of microorganisms chosen for the testing should reflect the potential risk. As the Ph. Eur. allows some flexibility in the experimental conditions and range of microorganisms, the materials and methods for testing, if different from the ones listed in Ph. Eur. 5.1.3., should be described in appropriate detail by the applicant, who must also validate the method to “ensure that any residual antimicrobial activity of the product is eliminated by dilution, filtration or by the use of a specific inactivator” in the recovery operation. The maintenance of the quantity of preservative (or the preservative efficacy if justified) throughout the period of the immunological veterinary medicinal product shelf life should be demonstrated.

2.4. Diluents

2.4.1. Definition

Annex I of Directive 2001/82/EC, Title II, Part 1.A states that: “Information on diluents needed for making the final vaccine preparation shall be included in the dossier. An immunological veterinary medicinal product is regarded as one product even when more than one diluent is required so that different preparations of the final product can be prepared, which may be for administration by different routes or methods of administration.” The diluent does not contain any active substance.

2.4.2. Data requirements

The data for production and control should follow the principles for IVMPs (Annex I, Title 2) where applicable. The dossier should provide the relevant data especially for:

- Qualitative and quantitative particulars
- Description of the manufacturing method

- Production and control of starting materials
- Control tests during the manufacturing process
- Control of the finished product
- Sterility
- Virucidal/bactericidal effect on the active substance by using the diluent to solve the active substance prior to titration
- Stability tests
- Starting materials used for the production of IVMPs for food producing species should comply with the current MRL legislation.

The IVMP for which the diluent is intended for should be fully tested for safety and efficacy. Provided the relevant studies are performed with the final product solved in the diluent, no separate studies on the diluent concerning safety and efficacy are required.

2.5. Purity of antigen harvest for inactivated vaccines produced on eggs (Bioburden)

For viruses grown in eggs, each batch of clarified virus harvest shall be tested for the amount of bacteria present and the value obtained shall be included on the batch test protocol. In general, it is stated that the production (harvest) process should ensure that the bioburden is as low as possible. Reduction of the bioburden and the validation of the inactivation procedures shall be considered not only for the vaccine antigen but also for the amount of bioburden present in the bulk prior to inactivation.

The maximum bioburden should be defined by the applicant, based on data from validation of inactivation and safety studies and it should be controlled in each harvest or bulk as an in process control.

2.6. Inactivation

Annex I of Directive 2001/82/EC states under Title II, Part 2, D. Control tests during the manufacturing process: "For inactivated or detoxified vaccines, inactivation or detoxification shall be tested during each production run as soon as possible after the end of the inactivation or detoxification process and after neutralisation if this occurs, but before the next step of production." Under E. Control tests on the finished product, it is mentioned that a test to verify inactivation shall be carried out on the product in the final container unless it has been conducted at a late stage in-process.

It is considered that a single test to confirm complete inactivation carried out at the stage after inactivation when detection of any residual live antigen is most likely should give sufficient assurance of complete inactivation and compliance with the pharmacopoeial standard.

Validation of the inactivation process of immunological veterinary medicinal products is subjected to the provision of data showing complete inactivation of the vaccine micro-organism. To this aim, according to Ph. Eur. monograph 0062, *Vaccines for veterinary use*, data on inactivation kinetics should be obtained using the selected method of inactivation. However a clear indication is only given concerning the time required for inactivation which, normally, should not exceed 67% of the duration of the inactivation process. It is considered that extrapolation of inactivation kinetics results (during a 1-step process) to higher pre-inactivation titres than those used in the corresponding validation studies

is not permitted. The maximum titre of the vaccine micro-organism capable to be inactivated by the selected method of inactivation should be then established based on the actual data obtained from inactivation kinetics studies.

2.7. Samples

Representative samples of all seed materials (e.g. subsequent passages), reagents, in-process materials and finished product shall be supplied to the competent authorities, on request.

3. Control on the finished product

The control tests on the finished product mentioned in the Annex I of Directive 2001/82/EC under Title II, Part 2. E shall normally be performed on each batch or sub-batch of vaccine produced. In the case of sub-batches which differ only due to their processing after bulk blending, for example in their filling session or vial size, some tests may be carried out on the final bulk or on one of the sub-batches, if justified.

It should be demonstrated that the subsequent procedure does not result in differences in test results and the results obtained from tests on the bulk can be reproduced on the sub-batch(es) of the finished product. For example, it may be expected that tests of potency of liquid inactivated vaccines could be done on the final bulk. On the other hand, tests for sterility must be carried out on each sub-batch.

3.1. Batch titre or potency

For a live vaccine, the titration of the active substance shall be validated according to the principles of the VICH GL1 "*Guideline on validation of analytical procedures: definition and terminology*" and VICH GL 2 "*Validation of analytical procedures: methodology*". An inactivated vaccine shall be shown to be of satisfactory potency using validated methods.

3.2. Preservatives – Identification and assay of excipients components

Tests for the concentrations of preservatives shall be carried out to show that these are in conformity with the limits set for the product. The concentration of preservative at release can be higher than at the end of the shelf life if the efficacy of the preservative has been demonstrated with the lower concentration. The composition of the product shall indicate the lower concentration of the preservative.

3.3. Safety tests

The Directive 2001/82/EC requests that an overdose safety test is performed on the finished product. As the Ph. Eur. monograph 0062 Vaccines for Veterinary Use does not request this test anymore, it is considered that the batch safety test is not mandatory as a control of the finished product.

3.4. Batch protocols

The batch protocols should be based on the templates issued by the European Commission and the European Directorate for the Quality of Medicines (EDQM) at the time the batch was produced.

4. Stability tests

Stability testing shall be carried out as specified in the Directive 2001/82/EC and in the European Pharmacopoeia monograph 0062 Vaccines for Veterinary Use on not fewer than 3 representative consecutive batches. The three consecutive production runs may be carried out on a pilot scale, providing this mimics the full-scale production described in the application. The sterility of the vaccine has to be proven at the end of the shelf life. This can be achieved by sterility testing or alternatives (e.g. test for container/closure integrity). Where bulk material is to be stored before formulation and final manufacturing, stability data should be provided.

III and IV Safety and efficacy tests

Animal welfare concerns should be taken into consideration in compliance with Directive 2010/63/EC when designing studies to test the safety and efficacy of IVMPs. Aspects to be considered include:

Personnel conducting the studies should be appropriately trained to detect signs of illness as well as behavioral changes in the test animals.

The method used to identify vaccinated and controls animals should involve the least harmful technique for the animals in the study.

The number of animals in the vaccinated and control groups should be sufficient to obtain statistically significant and clinically reliable results. However, for vaccination-challenge studies, the possibility of reducing the number of control non-vaccinated animals should be investigated as these animals will suffer disease and associated distress.

Mortality as an evaluation parameter in vaccination-challenge studies should be questioned whenever possible; humane endpoints are preferable. Moribund animals should be humanely killed.

1. Safety tests

Safety testing shall be carried out as specified in the Ph. Eur. general chapter 5.2.6 Evaluation of safety of veterinary vaccines and immunosera and in Directive 2001/82/EC. The vaccine to be tested shall be diluted in the recommended diluent, if appropriate.

2. Field trials

Safety and efficacy must be studied in field trials performed on a sufficient number of target species distributed in more than one premises.

Annex 1

Additional items, specific requirements for the production and control of immunosera and colostrum substitutes

This annex is intended to provide additional guidance on the type of data which should be included in applications for marketing authorisations for immunosera and colostrum substitutes. It is intended to supplement Directive 2001/82/EC and the general guideline.

The annex has not been prepared to give guidance for applications for products containing monoclonal antibodies and may not be applicable to such products.

Definitions

The definitions in the Ph. Eur. monograph “Immunosera for Veterinary Use” (01/2008/0030) apply together with the following additional definition:

Immunoserum – a veterinary medicinal product containing for example, polyclonal antibodies, or immunoglobulin fractions, or antibodies produced in eggs and used to provide passive immunity, through its immunoglobulin content.

Colostrum Substitute – a veterinary medicinal product for administration by the oral route to new-born animals to provide passive immunity, through its immunoglobulin content. It contains, for example, polyclonal antibodies, or immunoglobulin fractions, or antibodies produced in eggs.

Donor Animal – an animal which is kept for the production of immunoserum or colostrum or antibodies produced in eggs.

The donor animals may or may not have been actively immunised to boost the concentration of immunoglobulins to one or more specific antigens.

1. Starting materials

Preparation of the material containing the active ingredient

1.1 Donor animals

Donor animals should comply with the Ph. Eur. monograph “Immunosera for Veterinary Use 01/2008/0030.

Detailed information must be provided of the testing regime used to monitor the health status of the animals and this must include information on the test methods used and their validation.

1.2 Immunising antigen

Immunising antigen should comply with the Ph. Eur. monograph “Immunosera for Veterinary Use 01/2008/0030.

Wherever possible, the immunising antigen used should be a product with a marketing authorisation granted in the relevant Member State, in accordance with the requirements of Directive 2001/82/EC.

When an authorised product is used, it will be sufficient, in the dossier provided in support of the application for a marketing authorisation for the immunoserum or colostrum substitute, to provide

brief details of the immunising antigen (e.g. name, licence number, holder of the marketing authorisation, manufacturer(s) and the SPC).

Where the immunising antigen is not an authorised product the principles and the format of Directive 2001/82/EC and this guideline can be used as a guide for this.

For live organisms, for inoculation into a donor animal, information should also be provided on the safety of the organisms for the donor animal and it may be necessary to provide information on the rate of clearance of the organism from the material to be collected from the donor (e.g. where there may be a long lasting infection or a short time from immunisation to collection of material).

2. Finished product – batch testing

2.1 Sterility

The product shall be shown to meet the requirements of the Ph. Eur. for sterility and freedom from mycoplasmas unless it is a colostrum substitute to be administered orally, in which case it may contain not more than one saprophytic organism per dose.



Acts and Regulations concerning the Care and Use of Fish in Norwegian Research

**1st Edition
October 2005**

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Important comments on the document

Underlined text indicates direct web-links available in the electronic version, and for the paper version the web-addresses are provided at the end of the document. Some of the links are only available in Norwegian and then the Norwegian title is used. In some cases, links to both Norwegian and English versions are available.

We have interpreted the Acts and Regulations to the best of our knowledge and apologise for any misinterpretations. This report should not be used as a legal document.

The report is written in English and will be part of the study material for [FELASA category C courses](#) for fish researchers in Norway.

This document will be continuously updated and it is therefore important to include the edition number when referring to the document. If you have any suggestions for changes/corrections please contact us and we will consider it for the next edition.

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Introduction

This report is part of a project financed by the Norwegian Food Safety Authority to establish a platform for increasing implementation of “the 3 R’s” in Norwegian research.

The 3R’s

- **Replace** - replace animal experiments where possible with alternatives
- **Reduce** - reduce the number of experiments, and the number of animals in each experiment, to an absolute minimum
- **Refine** - refine experiments that have to be carried out, so that the animals undergo the minimum of discomfort, and such that the scientific quality is as high as possible

It is an ethical dilemma to use live animals in research, so the number of animals and their suffering should be kept to a minimum. The main aim of the project is therefore to define a 3R-strategy that will provide additional and improved research results per animal used in research.

Since over 90% of animals used in research in Norway are fish, the project is mainly focussed on these species. We have previously issued a report entitled [“An analysis of the fish used in research in Norway in 2003: Numbers, species and use”](#).

The aim of the present report is to provide an overview of Acts and Regulations governing the care and use of fish in research in Norway. This includes Regulations that demand the use of fish in the development of medicines (including fish vaccines), testing of chemicals and toxin diagnosis. Emphasis is also placed on how this legislation affects implementation of the 3 R’s.

A fourth R, Relevance, also needs to be questioned in accordance to Acts and Regulations. This is due to the fact that most fish used for research in Norway are used for testing of side-effects of fish-vaccines (batch testing). Even though the laboratory tests show little side-effects the problems with side-effects due to fish vaccines in the fish farming industry continues to be a large problem. The lack of consistency between the laboratory tests and the field results questions the relevance of the mandatory testing.

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Summary

- **There is a need for better definitions concerning fish welfare**
 - Researchers are still debating whether fish have the ability to suffer and this makes it difficult to define terms in the legislation. More knowledge in this area is needed, it should be emphasised that research will never provide a complete picture of how fish feel.
 - Acts and Regulations should be based on current assumptions on the needs of fish, including their perception of pain and suffering. Legislators must therefore be kept updated on the latest knowledge in this field.
- **Regulations for the care of fish in research**
 - The “Akvakulturdriftsforskrift” covers not only farmed fish but also fish in research facilities, and provides many details on their care. It is important to note that exemptions from this regulation can only be made by the Norwegian Food Safety Authority (Mattilsynet) and not by the Norwegian Animal Research Authority (Forsøksdyrutvalget).
 - The “Akvakulturdriftsforskrift” and a range of guidelines state that the health and welfare of the fish need to be monitored, but do not provide details on how the monitoring should be provided. A set of general guidelines is needed, and a separate paper on “Guidelines for health and welfare monitoring of fish in research” will be published by this Centre.
- **Regulations that demand the use of fish in research**
 - The European pharmacopoeia and other international regulations often state exactly how many fish that must be used, and how, for example, vaccine or chemical testing should be conducted. It is difficult to implement the 3 R’s in these regulations.
 - The end-point in fish models is often death, and many regulations do not allow refinements of the method. LC₅₀ tests are, for example, still used for testing chemicals on fish. Changes need to be made to allow more humane end-points, since this is an important part of the implementation of the 3 R’s.
 - The report [“An analysis of the fish used in research in Norway in 2003: Numbers, species and use”](#) concludes that most fish are used for batch testing of vaccines according to the European pharmacopoeia. This illustrates the impact of international regulations on the number of fish used in Norway.
- **Changes in the legislation**
 - The Norwegian Animal Welfare Act, the EU Directive 86/609 and the Council of Europe’s Convention ETS 123 are all currently being revised.
 - The Norwegian Food Safety Authority provides a [website](#) with updated Norwegian regulations. However, there is also a need for a website that is updated continuously covering the international regulations that govern the use of fish in research in Norway.

Main conclusions

- There is a need for implementation of the 3 R's in Regulations that make it mandatory to use live fish. For example, Regulations for the testing of fish vaccines and toxins do not in many cases allow any refinement of the method or reduction of the number of fish. The relevance of the mandatory tests also needs to be questioned.
- The lack of clear definitions of fish welfare has resulted in vague legislation that can be interpreted in many ways. Better definitions are necessary to ensure correct interpretation.
- A website with the latest science-based knowledge on the care and use of fish in research, together with the latest data on the ability of fish to feel pain would be of great help to fish researchers, technicians and legislators. There is also a need for an updated website on Acts, Regulations and guidelines related to research using fish.

The Animal Welfare Act

[The Norwegian Animal Welfare Act No. 73 of 20th December 1974 \(Dyrevernloven\)](#) and the Regulation relating to experiments with animals (*Forskrift om forsøk med dyr*) both include fish in their definitions of an animal. This is not the case in many countries. The Act is now being revised and is scheduled to be finalised in 2006.

The main principle and the scope of the Act according to Article 2 is as follows: “*Animals shall be treated well, and consideration shall be given to the instinctive behaviour and natural needs of animals, so that there is no risk of causing them unnecessary suffering.*” What is to be defined as “unnecessary” must be considered in each specific case according to current scientific data on, among other things, pain perception, on the purpose or aim of the study and on society’s ethical standards. For example, fishing for food is considered “necessary” and is therefore allowed, while “catch and release” of fish is not considered “necessary” as the purpose is for humans to enjoy a sport and it therefore [questioned](#) in Norway.

Researchers still debate whether fish have the [ability to suffer or not](#) and this makes the term “unnecessary suffering” vague when it comes to fish welfare (Rose 2002, Sneddon 2003). Better definitions and knowledge on fish welfare are therefore of major concern. In the last decade there has been an ever increasing focus on fish welfare issues, and in connection with the on-going revision of the Animal Welfare Act it is considered essential to implement new fish welfare legislation. The first step in the revision was to establish a working group. The Group’s mandate was to identify society’s attitude towards animal welfare, to establish new ethical standards, to describe how the different species of production and companion animals are kept, to identify important welfare issues for each species and to suggest new means for improving animal welfare and addressing the problems that had been identified. The proposal of the working group was presented in December 2002: “[Stortingsmelding nr. 12 \(2002-2003\) Om dyrehold og dyrevelferd](#)”. This Parliamentary Report on animal welfare was debated in the Norwegian parliament and approved by the Government in June 2003. In the Report it is explicitly stated that though there is still some doubt whether fish feel pain in a similar way as mammals, they must be treated as if they are capable of feeling pain and fear and thus are able to suffer.

Articles 21 & 22 in the current Animal Welfare Act state that animals cannot be used in research without a licence from a committee. The Committee is organised under The Norwegian Food Safety Authority and is called the Norwegian Animal Research Authority (NARA, *Forsøksdyrutvalget*). Today the NARA consists of 8 members who are appointed for a 4-year period. The secretary of the committee is the only full-time employee.

The Regulation relating to experiments with animals

[1996-01-15 No. 23 The Norwegian Regulation relating to experiments in animals \(Forskrift om forsøk med dyr\)](#) describes the work of the Norwegian Animal Research Authority (NARA) and how biological experiments shall be conducted. NARA approves laboratory animal facilities and personnel including a “responsible person” (*Ansvarshavende*) for each unit.

The “responsible person” alone approves of most experimental studies carried out at the unit and these approvals are later revised by NARA within a month. The regulation provides a list of experiments that can not be approved by the “responsible person” alone and that have to be approved directly by the NARA, for instance experiments that are assumed to cause “prolonged or significant pain”. The definition of “significant pain” for fish, however, is not defined. In 2003 only 36 fish of a total of about 400.000 fish used in Norwegian research were considered to suffer “significant pain” and all these fish had radio-transmitters surgically implanted in their abdomen. Challenge trials where fish are submitted to lethal doses of infectious agents were, for example, not defined as studies causing “significant pain”. The practice of one person at each unit making most decisions has been questioned and criticised and is currently under discussion.

Field studies outside laboratory units must always be approved directly by the NARA.

The same reporting criteria are used for all laboratory animals and no differentiation is made between different fish species or ages. Adult salmon are, for example, reported in the same statistics as cod larvae. The problems with vague definitions on how to report the number of fish being used have been highlighted in the report: [“An analysis of the fish used in research in Norway in 2003: Numbers, species and use” \(Knudsen et al. 2005\).](#)

The Norwegian Regulation states that housing and environmental conditions must satisfy the guidelines in Appendix A of the European Convention (ETS 123). As mentioned below this Appendix does not cover fish at present, but fish will be included in the revised version. The new National Animal Welfare Act in Norway along with the new Appendix A will create the need for a new or revised regulation governing the care and use of fish in research.

The European Convention (ETS 123)

Fish are included in the [European Convention for the “Protection of Vertebrate Animals used for Experimental and other Scientific Purposes” \(1986\)](#) and Norway was one of the first countries to sign the agreement. Species-specific regulations are provided in [Appendix A: “Guidelines for accommodation and care of animals”](#). The Appendix does not include fish today (October 2005), but a revision of the Appendix has started and the [new draft](#) includes fish. The draft provides general regulations to control parameters such as water quality, health, handling, transport and housing. Species-specific guidance on rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), tilapiine cichlids, zebra fish (*Danio rerio*), sea bass (*Dicentrarchus labrax*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*) and African catfish (*Clarias gariepinus*) will be available in the background document drawn up by a group of experts, but this is not yet available.

[Appendix B](#) describes how statistics on the number of animals used for experimental and other scientific purposes should be reported. The parties have agreed on some [changes in the statistics](#) (Daniel Cangemi, Secretary of the Conventional Committees on Animal Welfare, personal communication). The statistics from different countries vary greatly, making it difficult to compare data and they are therefore published separately for each country. For some reason the Norwegian data are not shown on the Council of Europe’s [web-site](#).

There is also a convention covering farm animals, the [European Convention for the Protection of Animals kept for Farming Purposes \(EST No. 87\)](#). Recommendations concerning cattle, sheep and other mammals have already been made and recommendations for farmed fish will

be discussed at a meeting in December 2005 (Daniel Cangemi, Secretary of the Conventional Committees on Animal Welfare, personal communication).

The EU Directive (86/609/EEC)

Norway is not member of EU, but EU Directives still have a major influence on our legislations through the EEA agreement. Most countries that have ratified the European Convention (ETS 123) are also members of the EU and therefore have to conform to “[The Council Directive 86/609/EEC of 24th November 1986 on the approximation of Acts, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes](#)”. In 2000, the European Union itself adopted the ETS 123, making it compulsory to adopt the same changes into the Directive, but this process requires the approval of the European Parliament. [The Directive is now being revised](#), but it will probably not include guidelines for accommodation and care.

The main object of the Directive is to ensure harmonisation of Acts concerning laboratory animals in the EU member states and ensure that the number of animals used and their suffering is kept to a minimum. It includes all non-human vertebrates except for foetal and embryonic forms. Fish are not mentioned in particular, but the general articles are suitable also for fish.

It is interesting to note the definition of “experiment”: *“any use of an animal for experimental or other scientific purposes which may cause it pain, suffering, distress or lasting harm, including any course of action intended, or liable, to result in the birth of an animal in any such condition, but excluding the least painful methods accepted in modern practice (i.e. 'humane' methods) of killing or marking an animal”*. The definition of ‘pain, suffering and distress’ in fish therefore becomes a major issue and should be defined in the new version of the Directive.

All 15 EU member states in 2001 agreed upon 8 tables on [“Statistics on the number of animals used for experimental and other scientific purposes”](#) (published in 2005). Hopefully this agreement will provide data that are comparable over time and between different countries. These statistics run in parallel to the statistics mentioned in Appendix B of ETS 123.

Acts and Regulations for farmed fish in Norway

[The Act of 14th June 1985 No. 68 relating to aquaculture](#) (*Oppdrettsloven*), §2 states “The term aquaculture means any activity involving the feeding or handling of live fish and shellfish for consumption, feed production, reproduction, stocking, including sea ranching, research or educational purposes.” Fish research is thereby defined as part of aquaculture. The Acts and Regulations governing aquaculture apply therefore also for fish in research.

[The Act of 1st Jan. 2004 No. 124 \(“The Food Act”, “Matloven”\)](#) involves all aspects of food production and animal health including fish. This new Act replaces many previous Acts including the “Fish Diseases Act” (*Fiskesykdomsloven*). The Food Act is the legal basis for

a number of regulations of relevance to fish research, and the most important ones are mentioned below.

[An overview of most Regulations](#) is presented on the website of The Norwegian Food Safety Authority. An English translation of all Acts and [Regulations related to farmed fish was last updated in 2003 \(also in English\)](#). However, many changes have been made over the last two years and these new Regulations have not yet been translated.

[1995-01-01 No. 99 “Forskrift om fortegnelse over sykdommer hos fisk og andre akvatiske dyr som omfattes av matloven”](#). This Regulation provides a list of fish diseases to which the Act is especially applicable. The list is adapted to Norwegian conditions and the diseases are divided into three groups: A, B and C.

[1990-02-05 No. 144 relating to instructions for A, B and C diseases \(English version in 2003\)](#)

This Regulation provides information on how detection of diseases in groups A, B and C are to be handled. Diseases in Group A are considered exotic in Norway, and thus any detection will result in stamping-out procedures (slaughter or destruction of all fish). Detection of diseases in Group B has to be reported to the authorities and different actions are then taken depending on the circumstances. The authorities also have the possibility of making general or local decisions on diseases in Group C.

[1999-12-20 No. 1310 relating to vaccination of domestic animals, wildlife, fish and other aquatic animals \(Vaksineforskriften\) \(English version in 2003\)](#) According to this Regulation, vaccination is allowed against diseases in Group C, but not against diseases in Group A and Group B, with the exceptions of furunculosis and infectious pancreatic necrosis (IPN).

[2004-12-22 No. 1785 “Akvakulturdriftsforskriften”](#) This Regulation has replaced several older ones, including no. 1397 The Hatchery Regulation (*Settefiskforskriften*), No. 1409 Operating and Disease Prevention Regulation (*Drifts- og sykdomsforskriften*), No. 278 Health Control Regulation (*Helsekontrollforskriften*) and parts of No. 509 Disease Control Regulation (*Sykdomsforskriften*). The Regulation is related to three Acts; The Food Act (No. 124), the Aquaculture Act (No. 68) and the Animal Welfare Act (No.73).

This Regulation provides guidelines both for monitoring of the health and welfare of the fish and also for water quality and other environmental factors. It states the minimum number of health inspections that are necessary and the number of fish that needs to be examined in relation to different categories of fish. Broodstock fish and young fish are, for example, to be given a minimum of 12 health examinations a year, while larger fish for food production need only 6 inspections. The monitoring of health and welfare is “risk based” (*risikobasert*) which opens for adjustments to the risk situation for the particular fish group at a given location. Monitoring and interpretation of risk factors therefore becomes a major issue.

The Norwegian Ministry of Fisheries and Coastal Affairs has issued [guidelines](#) to help people with the interpretation of the regulation (58 pages). Chapters 1, 2, 3 and 7 apply to fish in research. The guidelines state that exemptions from one or more of the provisions in the regulation may in exceptional cases be granted if there is a good reason for it. Furthermore it

is stated that it is not the intention of this Regulation to be a hindrance to research approved by the NARA but it is necessary to ensure all fish good welfare. The Norwegian Food Safety Authority is the only body that may grant an exemptions from the provisions given in the Regulation in pursuance of the Animal Welfare Act. It is also stated that certain types of research facilities will not be granted exemptions as they resemble commercial aquaculture farms.

The number of details in this Regulation is extensive, and both the Regulation and the guidelines need to be read in detail by everyone who works with farmed fish, including those involved in fish research. A table with acceptable values of some water quality parameters is provided for salmonids in freshwater and further tables for other fish species will hopefully be available in the future. Norms for expected mortality rates are provided for salmonids of different sizes and this is of major importance when an increase in mortality is to be evaluated.

As regards monitoring of fish welfare, §6 states that a sufficient number of personnel with necessary knowledge of fish welfare are needed. ‘Knowledge of fish welfare’ must be documented every fifth year (starting in 2010) using a plan approved by the Norwegian Food Safety Authority (not available yet).

The health monitoring must be carried out by authorised animal health personnel according to **The Act of 15th June 2001 No.75 (“[Lov om veterinærer og annet dyrehelsepersonell](#)”, *Dyrehelsepersonelloven*)**. As far as fish health is concerned, the Act equates veterinarians and fish health biologists (M.Sc. in Aquamedicine). Some exemptions to the types of health monitoring that can be performed by non-authorised personnel are also listed in the guidelines.

[2004-03-19 No. 537 “IK-Akvakultur”](#) states that the person responsible for an aquaculture establishment, including research facilities, is responsible for documentation that the establishment is run in accordance to all Acts and Regulations (*Internkontroll*). The one-page regulation is followed by three pages of guidelines which provide details on how this monitoring should be carried out. A [web-site](#) for additional help with implementation of “IK-Akvakulture” is also available in Norwegian. The Norwegian Food Safety Authority inspects the documentation mandatory by The Food Act (No. 124) and the Animal Welfare Act (No. 73), while The Directorate of Fisheries controls Regulations under The Aquaculture Act (No. 68).

Other Regulations

Regulations concerning field studies

Field studies outside approved laboratory facilities constituted 10% of all fish used in research in 2003. In these cases there are several Acts and Regulations concerning wild fish that must be followed.

[Act No. 47 15th May 1992 relating to salmonids and fresh-water fish etc.](#) protects wild species of salmonids and freshwater fish along with their habitats. §8 forbids importation of these fish species without permission from the government. The rest of the Act is mainly related to fishing regulations.

1991-07-04 No. 509 relating to prevention, control, and eradication of diseases in aquatic organisms mainly concerns wild fish since most of the clauses related to fish farming have been moved to no. 1785 “*Akvakulturdriftsforskriften*”. There are, however, still clauses covering disease eradication and control. The Regulation also addresses how to avoid spread of disease from one river or water system to another.

Regulations on import, export and movement of fish

1991-07-04 No. 509 relating to prevention, control, and eradication of diseases in aquatic organisms (Sykdomsforskriften), §19, regulates the importation of fish for use in research and states that they have to be proven free from diseases in Group A. Certificates must be issued by the official veterinary authority or other regulatory authorities at the place of departure and a number of other requirements are stated.

2003-10-14 No. 1239 “Forskrift om dyrehelsemessige vilkår ved omsetning og import av akvakulturdyr og akvakulturprodukter” (Omsetningsforskriften) provides additional regulations for the importation and movement of fish. Separate paragraphs cover importation from countries outside Europe and between different zones. This Regulation also includes zebrafish and other aquarium fish. Transport documents must include a health statement that the fish satisfy the requirements laid down in **The European Council Directive of 28th January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products (91/67/EEC)**.

2003-05-30 No. 661 describes a range of protection measures to be taken in connection with import and export of aquatic animals and products thereof ([English version in 2003](#)).

1997-12-20 No. 193 relates to the transport of aquatic organisms ([English version in 2003](#)). This Regulation states how fish can be transported and provides requirements for vehicles, tanks and other equipment used to move the fish.

1998-12-31 No. 1484: Forskrift om tilsyn og kontroll ved innførsel og utførsel av levende dyr, annet avlsmateriale og animalsk avfall innen EØS, og ved innførsel av levende dyr fra land utenfor EØS. This Regulation covers the supervision and control of import and export of live animals, other breeding material and animal waste within the EEA, and also covers the import of live animals from third-party countries ([English version in 2003](#)).

Regulations on disinfection

1997-02-20 No. 194 regarding the cleaning and disinfection of aquaculture sites etc. (*Desinfeksjonsforskriften*) ([English version in 2003](#)) states which disinfectants that can be used and how buildings, tanks and other equipments are to be cleaned and disinfected.

1997-02-20 No. 192 relating to disinfection of intake water and effluent water from aquaculture related operations (*Vannbehandlingsforskriften*) ([English version in 2003](#)). This Regulation states how water that is taken into or released from research stations is to be disinfected. For research stations using salmonids or freshwater fish the reduction requirements for the disinfection method on the water inlet is $3\log_{10}$ (99,9%) of *Aeromonas salmonicida subsp. salmonicida* and IPN-virus. There are no general rules for treatment of the water intake for marine fish. Research stations assigned to challenge trials in category A need to show a minimum of $5\log_{10}$ (99,999%) reduction of IPN-virus at the water outlet, while

stations only assigned to challenge trials in category B and C need $5\log_{10}$ (99,999%) reduction of *Yersinia ruckeri*.

Legislation on genetically modified fish

[**The Gene Technology Act 1993-04-02 No. 38 \(Genteknologiloven\)**](#) regulates the use of genetically modified animals in Norway.

[**2001-12-21 No. 1602 Forskrift om innesluttet bruk av genmodifiserte dyr**](#) provides detailed requirements for the housing and management of genetically modified organisms including zebrafish.

[**1998-11-13 No. 1066: Forskrift om transport og import av genmodifiserte organismer**](#) regulates transport and import of genetically modified organisms.

Testing of medicinal products

A large percentage of laboratory animals, including fish, are used for developing and testing of medicines and vaccines. The exact number of fish used for these purposes is not possible to obtain with today's official statistics.

[**1992-12-04 No. 132 Lov om legemidler m.v. \(Legemiddeloven\)**](#) and [**1999-12-22 No. 1559 Forskrift om legemidler \(Legemiddelforskriften\)**](#) are, respectively, the main Act and Regulation concerning pharmaceuticals in Norway and primarily govern sale and marketing.

2004-12-16 No. 1657 The Norwegian Pharmacopoeia contains the official standards for production and quality control of medicinal products. It consists of the [European Pharmacopoeia](#), 5th Edition, and "*Norske legemiddelstandarder 2004 med Addendum 2005*".

The European Pharmacopoeia includes several monographs for fish vaccines:

- A: 01/2002:0062 Vaccines for veterinary use
- B: 01/2002:1580 Vibriosis (cold-water) vaccine (inactivated) for salmonids
- C: 01/2002:1581 Vibriosis vaccine (inactivated) for salmonids
- D: 01/2004:1521 Furunculosis vaccine (inactivated, oil adjuvant) for salmonids

Monograph A describes general requirements for production control of vaccines for animal use, including fish vaccines. It focuses on consistent and safe production without, among other things, contamination. Monographs B-D provide more details for safety and potency testing of these particular vaccines.

Safety tests

Safety testing is in principle carried out by administering a double dose of vaccine to the fish and then observing the animals for side-effects for 21 days. A minimum of 50 fish of each species is used and three different batches of vaccines need to be tested. A minimum of 150 fish per species for which the vaccine is intended is therefore needed.

Potency tests

Potency testing is performed by challenging a minimum of 100 vaccinated and 100 unvaccinated fish by injection of the virulent microorganism. The cumulative percentage mortality in the vaccinated group of fish is registered when 60% of the control fish are dead. The relative percentage survival (RPS) is calculated and should be not less than 90% in monograph B, 75% in C and 80% in D.

Most of the vaccines used on salmonids in Norway today are polyvalent (often [4-6 antigens](#) per vaccine) and a potency test is necessary for each antigen (200 fish per antigen). Potency testing of a hexavalent vaccine therefore requires a minimum of 1,200 fish. The testing described is performed a limited number of times during development of the vaccine in order to document safety and potency of the product.

The challenge models required by the monographs often involve injection of the challenge-organisms. These models are often not the most suitable methods for documentation of the effect of the vaccine, thus in order to evaluate efficacy during development of products, additional bath or cohabitation models may need to be performed (Kjersti Gravningen, personal communication).

Batch tests

Batch testing, which is performed on each production batch of the vaccine, employs 10 fish for safety testing and 60 fish (30 vaccinated and 30 control fish) per antigen for potency testing. Only the most sensitive fish species for which the vaccine is intended need to be tested. A minimum of 370 fish (360 for potency and 10 for safety) is therefore needed per batch for a hexavalent vaccine.

The size of a batch depends, among other things, on the production system and success rate, and may vary from 100-500 litres. The vaccines used for salmonids normally contain 10,000 doses per litre. It is important to note that the vaccines tested in Norway may be used in other countries such as Chile, Ireland and Canada, while the vaccines used in Norway may be tested in other countries such as the Netherlands (Intervet Norbio) and Canada (Scanvacc).

The number of batches that need to be tested in a final bulk production may be reduced by performing tests to show stability of the production of at least 10 batches (**Stability testing**) according to the Position Paper “Data requirements for removing the target animal batch safety test for immunological veterinary medicinal products in the EU” ([\(EMA/CVMP/865/03/Final\)](#)).

The challenge trials for batch potency testing can be replaced by validated tests based on antibody response in the fish. In this case, 25 fish are vaccinated and compared to 10 unvaccinated fish. The antibody level must be as high as the results from batches showing adequate potency results by challenge trials. These serological tests may therefore reduce the number of fish needed for testing of a batch of a hexavalent vaccine from 360 to 35.

[The European Medicines Agency](#) (EMA) provides useful guidelines to the European legislations in this field like for instance:

- Guideline on adjuvants in vaccines ([EMA/CVMP/VEG/17/03/2004](#))
- Guideline on statistical principles for veterinary clinical trials ([EMA/CVMP/816/00-Final](#))

- [Guidelines for production and control of immunological veterinary medical products](#)
Volume 7B pages 91-97 provides specific requirements for safety, efficacy and potency testing of live and inactivated vaccines for fish including batch testing. An appendix provides a list of infectious agents of which farms providing fish for the trials must be free.

[VICH GL9 \(GCP\) \(2000\) Good Clinical Practice.](#) This is an international guideline (EU, Japan and USA) from the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicine Products (VICH). It provides guidance on how to design and conduct clinical studies of veterinary products, and states which documents that need to be obtained before, during and after clinical testing. Inspections should be made by a relevant regulatory authority, which in Norway is the Norwegian Medicines Agency (*Statens legemiddelverk*). This agency has provided a Norwegian guideline for clinical testing of vaccines for fish, [“Retningslinjer vedrørende klinisk utprøving av vaksiner til fisk”](#).

[1996-06-28 No. 693](#) “*Forskrift om forskrivning, tilvirkning og distribusjon m.v. av medisinfôr til dyr, fugler, fisk og andre akvatiske organismer*” relating to prescription, manufacturing and distribution etc. of medicated feed to animals, birds, fish and other aquatic organisms ([English version in 2003](#)). Involves medicated feed for all animals including fish.

Testing of chemicals and toxins

EU is working on new legislation for approval of chemicals, called the “Registration Evaluation and Authorisation of Chemicals” ([REACH](#)). Norwegian legislation will have to adapt to this new EU Directive and this may influence the number of laboratory animals required for chemical testing.

We have only found one Norwegian legal document (*Aktivitetsforskriften*) that makes it mandatory to use live fish for the testing of chemicals and toxins. International regulations that concern animal testing in Norway have not been investigated in the process of compiling this document.

In the Norwegian statistics for the use of animals in research, testing of chemicals and toxins is reported in category H (2.4) “Protection of human, animal and environment by testing of toxicity or safety, including testing for products and equipment for use in veterinary- and human medicine.” The number of fish used in relation to the various legislative documents or the various tests is therefore not known.

Methods for testing chemicals and toxins may be assigned by ISO, OECD (The Organisation for Economic Cooperation and Development) or OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic). Free copies of the [OECD Guidelines](#) for the Testing of Chemicals can be downloaded from the Internet, after application for a free 7-day password.

OECD Guidelines for testing of chemicals on fish:

No. 203 Fish, Acute Toxicity test

The aim of the test is to determine the concentration which kills 50% of the fish (LC₅₀). Six

freshwater fish species are recommended for the test, including zebrafish and rainbow trout. Preparations for replacing this test with a fish embryo test are in progress and will be submitted to OECD in late 2005 (Braunbeck *et al.*, 2004). [Braunbeck *et al.* \(2004\)](#) also provide a good overview (Figure 15) of the terminology used for eggs, embryos and larvae of fish.

According to Gunvor Knudsen, who examined all applications for the use of fish in [Norwegian research in 2003](#), most fish for toxicity testing were used in accordance with OECD 203.

No. 204 Fish, Prolonged Toxicity test

This test is used instead of no. 203 when appropriate. The aim of the test is to determine threshold levels of lethal effect and levels of observed effect, which may include among other things abnormal swimming behaviour and changes in body weight. The same six fish species as in 203 are recommended.

No. 210 Fish, Early-life Stage Toxicity test

The aim in this study is to estimate the “lowest observed effect concentration” (LOEC) and “no observed effect concentration” (NOEC). The test begins with placing fertilised eggs in the test chamber and continues until the fish are feeding. Mortality, deformities, abnormal behaviour and other parameters are registered. Five fish species are recommended for this test (Appendix 3), but an additional list of 15 well-documented species is also listed as possibilities (Appendix 5). These include several species of salmonids.

No. 212 Fish, Short-term Toxicity test on Embryo and Sac-fry Stages

As in no. 210, the aim of this test is LOEC and NOEC. Cod and other fish species with high mortality rates in the control groups cannot be used in test no. 210. Test 212 is a possibility for these fish species due to the short timespan. The sensitivity is less than in no. 210.

No. 215 Fish, Juvenile Growth test

The aim of this test is to estimate the concentration that would cause an x% variation in growth rate (EC_x), *i.e.* EC_{10} or EC_{25} . Alternatively, LOEC and NOEC may be determined. The fish are simply measured before and after exposure to the substance and compared to a control group. Rainbow trout is the only recommended fish species in this test, but zebrafish and medaka can be used (Appendix 2).

No. 305 Bioconcentration: Flow-through fish test

The test consists of two phases: the exposure (uptake) period (28 days) and post-exposure (wash-out). Fish groups are exposed to the test substance in different concentrations for 28 days and then moved to clean water. Concentrations of the substance are measured in the fish and in the water at different time points. Both freshwater and marine fish have been used.

2002-07-16 no. 1139 Forskrift om klassifisering, merking m.v. av farlige kjemikalier. This is the Regulation for classification of dangerous chemicals. Methods for animal testing are provided in [Appendix V of EU-directive 67/548/EEC](#). Method C1 is a copy of OECD test number 203, C13 - OECD 305, C14 - OECD 215 and C15 - OECD 212. OSPAR Harmonised Offshore Chemical Notification Format (HOCNF) documentation is needed for all chemicals.

2001-09-03 no. 1157 Forskrift om utføring av aktiviteter i petroleumsvirksomheten

(Aktivitetsforskriften), § 56, states how chemicals from the petroleum industry are to be tested on algae (*Skeletonema costatum*), copepods (*Acartia tonsa*), mud shrimp (*Corophium volutator*) and/or fish. The fish and shrimp test must be performed according to [“OSPAR](#)

[Protocols on Methods for the Testing of Chemicals Used in the Offshore Industry, 1995”](#).

This method is a modified version of the LC₅₀ OECD test no. 203 “Fish Acute Toxicity test”. The main modification is adaptation to two marine fish species: turbot (*Scophthalmus maximus*) and sheepshead minnow (*Cyprinodon variegatus variegates*).

Petroleum chemicals are first to be tested on copepods and algae, and only chemicals with a low toxicity to these organisms need to be further tested on fish. The “OSPAR Protocols on Methods for the Testing of Chemicals Used in the Offshore Industry (1995) will furthermore be updated in 2005 to limit testing on fish (Ann Mari Vik, Norwegian Pollution Control Authority (*Statens forurensningstilsyn*), personal communication).

LC₅₀ testing on mammals (OECD test no. 401) is no longer allowed. “Acute Oral Toxicity tests” are used instead (no. 420 Fixed Dose Procedure (FDP), no. 423 Acute Toxic Class Method (ATC), no. 425 Up-and-Down Procedure (UDP), Botham 2002). These new tests also involves testing on laboratory animals, but the number of animals and suffering have both been drastically reduced. These tests are often referred to in the mass media, and it is therefore important for fish researchers to notice that these are all tests on mammals and do not concern fish.

Fish screening assays for the detection of endocrine substances

Substances with endocrine effects (“endocrine disruptors”) may result in several changes in fish, that can be detected by a variety of different methods. The easiest test is to look for changes in the behaviour or morphology of the fish. [OECD has provided a review paper on these fish assays](#). This review paper will most likely become the background material for future OECD guidelines. The review also includes an introduction to endocrinology in fish which can be useful for most fish researchers. Three fish species are used in these tests: the fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The review also includes general information about these fish species and Table 3.1 provides pros and cons in the choice between them that is also useful in other fish models.

Guidelines

Guidelines for the care and use of fish in research, along with monitoring and reporting of health and welfare, are sparse compared to those available for mammalian laboratory animals. It is worth noting that there are more fish species than all other vertebrate species combined. Despite the fact that fish are studied in almost all biological disciplines (Powers, 1989), most guidelines for the care and use of fish in research are general documents that encompass all fish species in all types of research ([DeTolla et al. 1995](#); Casebolt et al., 1998; [CCAC](#)). There is a great need for more species-specific guidelines and in some cases research disciplines may require particular guidelines (http://zfin.org/zf_info/zfbook/zfbk.html).

Researchers have an ethical obligation to extract as much knowledge as possible from each animal used. Lack of standardisation of parameters such as genotype, water quality and handling procedures in fish research often lead to incomparable results and therefore unnecessary use of large numbers of fish. Harmonisation of health and welfare monitoring is one important factor in the standardisation process and a separate paper on this matter will therefore be published by this Centre.

Monitoring of fish welfare was included in a large report to the Norwegian Research Council in 2005 entitled "[*Forskningsbehov innen dyrevelferd i Norge*](#)". The report provides a good overview of the ability of fish to feel pain and stress. Common welfare and diseases problems are discussed, and there is a chapter on indicators for monitoring of fish welfare. Today's indicators such as behaviour and health are mentioned, together with discussions on possible welfare indicators that may be available in the future, such as physiological and molecular parameters.

Another Norwegian report on monitoring of fish welfare will soon be published on www.mattilsynet.no with the title "*Dyrevelferd i akvatisk dyrehold - herunder fremtidens dyrehold*". This report focuses especially on how fish welfare can be monitored either directly by examining the fish or indirectly by monitoring the environment. The report will focus on need for "on-farm" indicators to document the well-being of the fish.

The Fisheries Society of the British Isles (FSBI) has published a very thorough [briefing paper](#) on fish welfare that provides several definitions of welfare criteria in fish. The paper does not state what is acceptable and what is unacceptable, but provides a good overview of background knowledge. It also provides information on acute and chronic stress responses in fish and look at ways of measuring fish welfare.

The Office International des Epizooties (OIE) is an international organisation for animal health with 167 member states, including Norway. The Aquatic Animal Health Standard Commission (AAHSC) is responsible for fish health issues applied by the "Aquatic Animal Health Code" which from 2005 includes fish welfare. Two *ad hoc* groups, led by Dr. Tore Håstein from Norway, are at the moment working on guidelines for transport and slaughter of fish.

The organisation FELASA has already provided guidelines for the health monitoring of other laboratory animals (Rehbinder *et al.*, 1998; Nicklas *et al.*, 2002) and hopefully in the future guidelines will be available also for fish. An overview of guidelines and other resources on the care and use of fish in research is provided on <http://oslovet.veths.no/fish>.

Links and references

English translations of all Norwegian laws and regulations related to fish farming (2003): <http://dyrehelsetilsynet.mattilsynet.no/dyrehelse/publikasjoner/engelskutgaveaugust2003.pdf>

The Norwegian Animal Welfare Act and Regulation governing animal experimentation: <http://www.fdu.no/fdu/regelverk/>

ETS 123, new Appendix A: [http://www.coe.int/T/E/Legal_affairs/Legal_co-operation/Biological_safety_use_of_animals/Laboratory_animals/GT%20123%20\(2004\)%201%20Appendix%20A%20final%20for%20adoption%20DRAFT.pdf](http://www.coe.int/T/E/Legal_affairs/Legal_co-operation/Biological_safety_use_of_animals/Laboratory_animals/GT%20123%20(2004)%201%20Appendix%20A%20final%20for%20adoption%20DRAFT.pdf)

ETS 123, Appendix B: <http://conventions.coe.int/Treaty/EN/Treaties/html/123-B.htm>

ETS 123, revised version of Appendix B: http://www.coe.int/T/E/Legal_affairs/Legal_co-operation/Biological_safety%2C_use_of_animals/Laboratory_animals/Res%20interpretation.asp#TopOfPage

EU Directive 86/609/EEC: <http://europa.eu.int/eur-lex/lex/LexUriServ/LexUriServ.do?uri=CELEX:31986L0609:EN:HTML>

EU Directive 86/609/EEC, Revision:

http://europa.eu.int/comm/environment/chemicals/lab_animals/revision_en.htm#Next%20Steps

Statistical data from the EU:

http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/com/2005/com2005_0007en01.pdf

Act of 14 June 1985 No. 68 relating to aquaculture (*Oppdrettsloven*):

<http://www.ub.uio.no/ujur/ulovdata/lov-19850614-068-eng.pdf>

Act No 47 of 15 May 1992 relating to salmonids and fresh-water fish etc.:

<http://www.ub.uio.no/ujur/ulovdata/lov-19920515-047-eng.pdf>

EU directive 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products (91/67/EEC):

http://europa.eu.int/smartapi/cgi/sga_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=en&numdoc=31991L0067&model=guichett

The European Pharmacopoeia: <http://online.pheur.org/entry.htm>

The European Medicines Agency: <http://www.emea.eu.int/hums/general/contacts/CVMP.html>

The rules governing medicinal products in the European Union, Volume 7B:

<http://pharmacos.eudra.org/F2/eudralex/download/volpdf/vol7/vol7ben.pdf>

VICH GL9 (GCP) (2000): http://vich.eudra.org/pdf/2000/GL09_st7.pdf

REACH: <http://europa.eu.int/comm/enterprise/reach/overview.htm>

OECD-library: <http://miranda.sourceoecd.org/vl=2187489/cl=115/nw=1/rpsv/home.htm>

Braunbeck *et al.* 2004: http://www.altex.ch/pdf/artikel/altex_2_2005_Braunbeck.pdf

67/548/EEC Annex 5: <http://ecb.jrc.it/testing-methods/>

OSPAR protocol for fish and shrimp testing:

http://www.ospar.org/documents/dbase/publications/p00037_Parcom%20Protocols.pdf

OECD Review on fish screening assays for the detection of endocrine substances:

[http://appli1.oecd.org/olis/2004doc.nsf/linkto/env-jm-mono\(2004\)18](http://appli1.oecd.org/olis/2004doc.nsf/linkto/env-jm-mono(2004)18)

DeTolla *et al.*: http://dels.nas.edu/ilar_n/ilarjournal/37_4/37_4Guidelines.shtml

CCAC guidelines:

http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Fish/Fish%20Guidelines%20English.pdf

FSBI briefing paper on fish welfare (2002): <http://www.le.ac.uk/biology/fsbi/welfare.pdf>

Links to Regulations available only in Norwegian

Årsrapporter fra Forsøksdyrutvalget: <http://www.fdu.no/fdu/om/dbaFile3843.html>

Matloven: <http://www.lovdatab.no/cgi-wift/ldles?doc=/all/nl-20031219-124.html>

Mattilsynets liste over forskrifter: <http://www.mattilsynet.no/fisk/regelverk>

Forskrift om liste over fiskesjukdommer:

<http://www.lovdatab.no/cgi-wift/ldles?doc=/sf/sf/sf-19950101-0099.html>

Vaksineforskriften:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19991220-1310.html>

Instrukser for A, B, C sykdommer:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19900205-0144.html>

Akvakulturdriftsforskriften: <http://www.lovdata.no/for/sf/fi/xi-20041222-1785.html>

Merknader til Akvakulturdriftsforskriften:

http://www.mattilsynet.no/multimedia/archive/00012/Merknader_til_forskr_12075a.pdf

Lov om veterinærer og annet dyrehelsepersonell: <http://www.lovdata.no/all/nl-20010615-075.html>

IK-Akvakultur forskriften med merknader:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-20040319-0537.html>

Web-site for hjelp ved innføring av IK-Akvakultur:

http://62.113.154.12/ik_veileder

Forskrift om forskrivning, tilvirkning og distribusjon m.v. av medisinfôr til dyr, fugler, fisk og andre akvatiske organismer:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19960628-0693.html>

Forskrift om forebygging, begrensning og utrydding av sykdommer hos akvatiske organismer:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19910704-0509.html>

Omsetningsforskriften:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-20031014-1239.html>

Forskrift om særskilte beskyttelsestiltak ved innførsel av akvatiske dyr og produkter av disse:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-20030530-0661.html>

Forskrift om transport av akvatiske organismer:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19970220-0193.html>

Forskrift om rengjøring og desinfeksjon av akvakulturanlegg m.v.:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19970220-0194.html>

Vannbehandlingsforskriften:

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-19970220-0192.html>

Genteknologiloven: <http://www.lovdata.no/all/hl-19930402-038.html>

Forskrift om innesluttet bruk av genmodifiserte dyr (dyreforskriften):

<http://www.lovdata.no/for/sf/ho/xo-20011221-1602.html>

Forskrift om merking, transport, import og eksport av genmodifiserte organismer:

<http://www.lovdata.no/for/sf/md/xd-20050902-1009.html>

Legemiddeloven: <http://www.lovdata.no/all/hl-19921204-132.html>

Legemiddelforskriften: <http://www.lovdata.no/for/sf/ho/xo-19991222-1559.html>

Retningslinjer vedrørende klinisk utprøving av vaksiner til fisk:

<http://www.legemiddelverket.no/klut/retningslinjer-fiskevaksine.htm>

Forskrift om utføring av aktiviteter i petroleumsvirksomheten (aktivitetsforskriften):

<http://www.lovdata.no/cgi-wift/ldles?doc=/sf/sf/sf-20010903-1157.html>

Rapport om Forsknings behov innen dyrevelferd i Norge:

http://www.forskningsradet.no/CSStorage/Flex_attachment/82-02156-4%20Dyrevelferd.pdf

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The potential to increase use of the 3Rs in the development and validation of fish vaccines

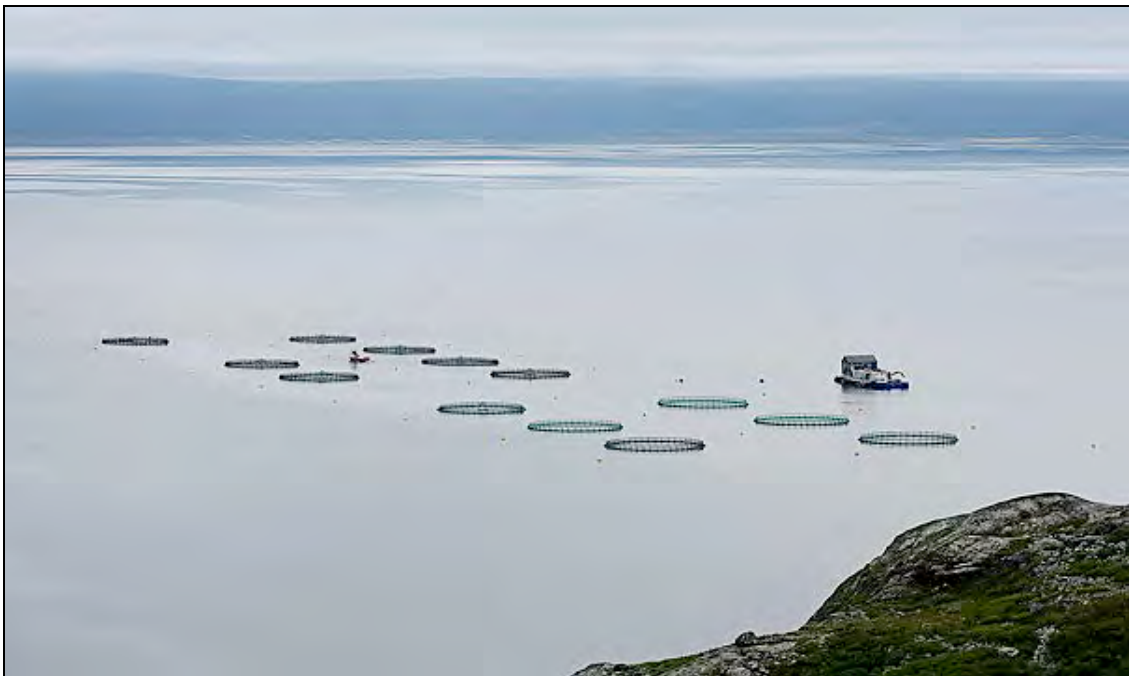


Photo: Marius Fiskum

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1. Background for the report

Vaccination of animals is an essential element in intensive farming systems to prevent disease outbreaks, thereby ensuring an economically viable industry and improved standards of animal welfare.

Large numbers of animals are used in the development and validation of vaccines for the fish farming industry. For this reason, Norecopa, the Norwegian Consensus-Platform for Replacement, Reduction and Refinement of animal experiments, commissioned a critical evaluation of the current requirements for the development and testing of vaccines in fish. The authors of this report were asked to evaluate the quality and necessity of the information derived from the techniques in use today, and to assess the potential for:

1. Replacing some of the *in vivo* testing with *in vitro* methods
2. Reducing the number of fish used in the various phases of development of fish vaccines.

Fish have dominated the Norwegian research animal statistics for many years. From 2005-2008 fish constituted 87-89% of all animals defined as research animals [1]. The number of animals used in research is a politically important but multifaceted issue, with strong arguments on the one hand for reducing numbers, to minimise animal suffering, and on the other hand the desire to maintain a leading position in cutting-edge research.

In 2009, fish constituted 97% of the 1.8 million research animals used in Norway. Salmon comprised nearly 20% of all research animals used that year and as much as 88% in 2008, largely due to vaccine trials. In addition, over 2 million animals were used in research in 2009 for purposes that fall outside the strict definition of a research animal – 97% of these were fish. In 2007, 3.4 million animals were used in research in Norway: 2.9 million of these were salmon used in one full-scale vaccine trial [1].

In 2011 ECVAM (European Centre for the Validation of Alternative Methods) published an extensive report entitled “Three Rs approaches in the production and quality control of fish vaccines” [2]. The recommendations of the ECVAM report are generally supported by the present authors.

Background information in chapters 3 and 4 is based on the book “Vaksinasjon av dyr [5].

2. Summary with main recommendations

The main recommendations of this report can be summarized as follows:

- In the documentation phase, and especially in the post-licensing phase, *in vivo* challenge tests can be substituted by antibody *in vitro* tests.
- The fish vaccine industry should be requested to validate the *in vitro* test for potency testing of furunculosis vaccine.
- The use of the Response Surface Pathway (RSP) during dose titration studies has the potential to reduce the number of test animals by 70% without loss of information.
- Statistical methods should be chosen with care and justified in potentially painful procedures.
- Well-defined and trial specific humane endpoints in vaccine development and validation need to be developed and used.
- Reducing the genetic variation within farmed fish breeds would also reduce the number of fish required for vaccine development and validation.
- The vaccine industry, regulatory authorities and scientific institutions should emphasize replacement in the development of vaccines for aquatic animals
- A number of fundamental factors related to equipment, procedures, animals, and staff need to be in place in order to obtain refinement and reduction.
- The recommendations in the ECVAM report “Three Rs approaches in the production and quality control of fish vaccines” [2] are generally supported.

3. Vaccines – a short introduction

3.1. What is a vaccine?

The English Oxford dictionary defines a vaccine as ‘an antigenic substance prepared from the causative agent of a disease or a synthetic substitute, used to provide immunity against one or several diseases’. A slightly different definition can be found in the European Pharmacopoeia: ‘Vaccines for veterinary use are preparations containing antigenic substances and are administered for the purpose of inducing a specific and active immunity against disease provoked by bacteria, toxins, viruses, fungi or parasites’ [3]. In other words, a vaccine is a biological entity that induces immunity to a specific disease.

A vaccine often contains an agent that resembles the causative agent, either in an attenuated or killed form. The administration of such an agent stimulates the body’s immune system, which in turn leads to inactivation of the foreign material (antigen). The immune system will in addition remember the antigen, which results in a more efficient antibody response at subsequent exposures.

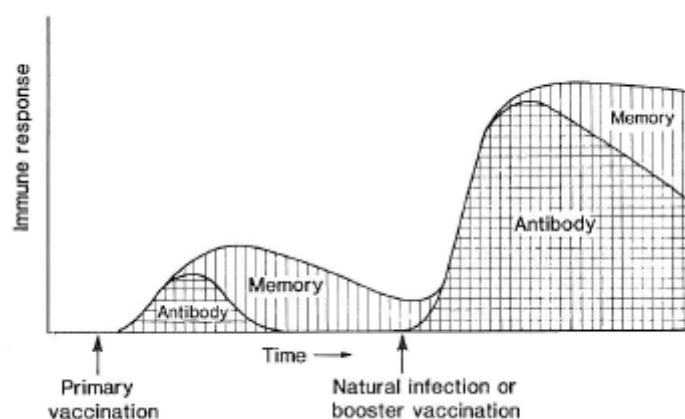


Figure 1. The immune response following primary vaccination and natural infection/booster vaccination [4].

The history of vaccination comprises efforts within both veterinary and humane medicine. The term “vaccination” stems from the work of the English doctor Edward Jenner (1749-1823), who discovered that people who had been infected with cow pox were immune to the more virulent human disease of smallpox. In May 1796 he rubbed pus from cattle with cow pox into the skin lesions of eight-year-old James Phipps who subsequently became immune. Hence, the word vaccine is derived from the Latin *vacca*, meaning cow.

Vaccines can be either *prophylactic* (to prepare the immune system for future infections) or *therapeutic* (to boost the immune system during an infection). The

focus of this report is on prophylactic vaccines since these are the ones of relevance to the fish farming industry.

3.2. Inactivated vaccines

Inactivated vaccines contain micro-organisms or products from these, that have been inactivated or killed. Inactivated bacterial vaccines are often referred to as bacterins. It is important that the inactivation is carried out in such a way that the immunostimulating abilities of the vaccine are maintained. If the denaturation process is too strong, these capacities will be weakened or absent.

Inactivated vaccines are most commonly administered by injection. For a full description of methods of administration, see section 4.4. The injection stimulates an antibody response dominated by Immunoglobulin G (IgG). To reach a satisfactory level of immunity following vaccination with inactivated vaccines, two doses may be necessary, with an interval of a few weeks (preferably between four and six). Optimal immunity is usually developed two to four weeks after the second vaccine dose. Inactivated vaccines generally require revaccination from time to time, typically every six to twelve months.

In most Western countries, including Norway, inactivated vaccines are preferred to live vaccines whenever possible, for safety reasons. Since the micro-organisms have been killed or inactivated, and in addition the vaccine contains preservatives, there is very little chance of spreading disease.

3.3. Attenuated (live) vaccines

A live vaccine generally consists of micro-organisms that are weakened to such a degree that they do not cause disease, but still evoke an immunological reaction. Vaccination with a live vaccine is in reality a controlled infection. The immune response following an attenuated vaccine will therefore bear more resemblance to a real infection. Live vaccines will also stimulate the development of cellular immunity more strongly than inactivated vaccines. Following vaccination with a live vaccine, immunity tends to be greater and more long-lasting than with inactivated vaccines. In many cases, it may also suffice with one application to achieve protective immunity.

3.4. A comparison between inactivated and live vaccines

There is an ongoing debate about the pros and cons of using live and inactivated vaccines. What is the better choice will vary between infections and will also depend on the nature of the infectious agent, the pathogenesis of the disease and the immune status of the animal. The prevalence and impact of the disease, and economic factors, must also be taken into account.

Table 1. A comparison of the effect, safety and practicality of inactivated and living vaccines [5].

Effect	Inactivated	Attenuated
Duration of immunization		+
Development of early immunity		+
Simulation of cellular immunity		++
Stimulation of local immunity		+
Protection against disease and infection		+
Affected by maternal antibodies		+
Need for adjuvant		+
Safety		
Pathogenicity	+	
Spread of infectious agent	+	
Recombination with feline virus	+	
Pathogenicity for other species	+	
Contamination with other agents	++	
General side effects	+	
Local side effects		+
Effect on foetus	+	
Incomplete inactivation		+
Practicalities		
Need for revaccinations		+
Price		+
Stability	+	
Need for solvents	+	

+ advantageous for the vaccine type

++ significantly advantageous for the vaccine type

3.5. New vaccine techniques

In addition to inactivated and attenuated vaccines, there are a number of recombinant vaccines, including subunit, gene-deleted, vector and DNA vaccines. These emerging techniques will not be discussed in this report.

3.6. Adjuvants

Most vaccines used today, both in the human and veterinary field, are added an adjuvant to enhance and prolong the immune response following vaccination. The adjuvant gives the vaccine a depot effect causing the organism to be exposed to antigens for a longer period of time leading to an enhanced immune response. Some adjuvants lead to quicker and more long-lasting immune reactions, while others control the immune response in certain ways, making it possible to promote the humoral or cellular part of the immune response.

All adjuvants cause adverse side-effects. Some are negligible like swelling, while others cause local adhesions and granulomas at the site of injection.

Adjuvants are generally categorized into three groups:

- Chemical substances like mineral salts or oils
- Bacterial products like components from mycobacterium, yeast cells or toxins
- Plant products like saponins or vegetable oils

3.7. Methods of vaccine administration

3.7.1. Injection

A common way to vaccinate fish is by injection. In Norway both manual and automated vaccination techniques are used. When fish are mechanically vaccinated, it is important that the machine is set correctly and supervised continuously in order to correct errors immediately. Both the site of injection and needle length are important, to ensure that the vaccine is deposited in the abdominal cavity and not in internal organs or muscles. The quality of vaccination is therefore best if the fish are roughly the same size. Sorting of fish is therefore important to achieve a good result.

Following the success of adjuvant vaccines against furunculosis, vaccination of fish in Norway occurs almost exclusively by intraperitoneal injection.

Vaccine administration by injection	
Advantages	Disadvantages
Generally good effect	High work load
Low vaccine consumption	Handling and anaesthetising the fish is stressful for the fish
(Life) long protection when administered with adjuvant	Local reactions at the site of injection due to adjuvant
Usually no need for double doses with a few weeks' interval when administered with adjuvant	Risk of self-injection by personnel with subsequent infections
No need for revaccination after release into the sea	Quality dependent on the vaccinators
	Unsuitable for small fish

3.7.2. Immersion

During the first years of fish vaccination, dip and bath vaccinations were commonly used. Today, immersion vaccination is only used for immunization of fish that are too small to be vaccinated by injection.



Vaccine administration by immersion	
Advantages	Disadvantages
No need for anaesthetics or handling – less stressful	Large amount of vaccine required
Suitable for mass vaccination of all sizes of fish	Lower level of protection
Lower labour costs	Shorter durability of vaccine
Less risk to personnel	

3.7.3. Oral

Oral administration is a cost-efficient and easy way of administering a vaccine. The technique has, however, a number of disadvantages.

Vaccine administration per os	
Advantages	Disadvantages
Can be mixed with feed	Large quantities of antigen required
Easiest method for mass vaccination of all sizes	Requires all fish to be feeding
Lower labour costs	Protection generally weak and of short duration
Low stress levels for fish	

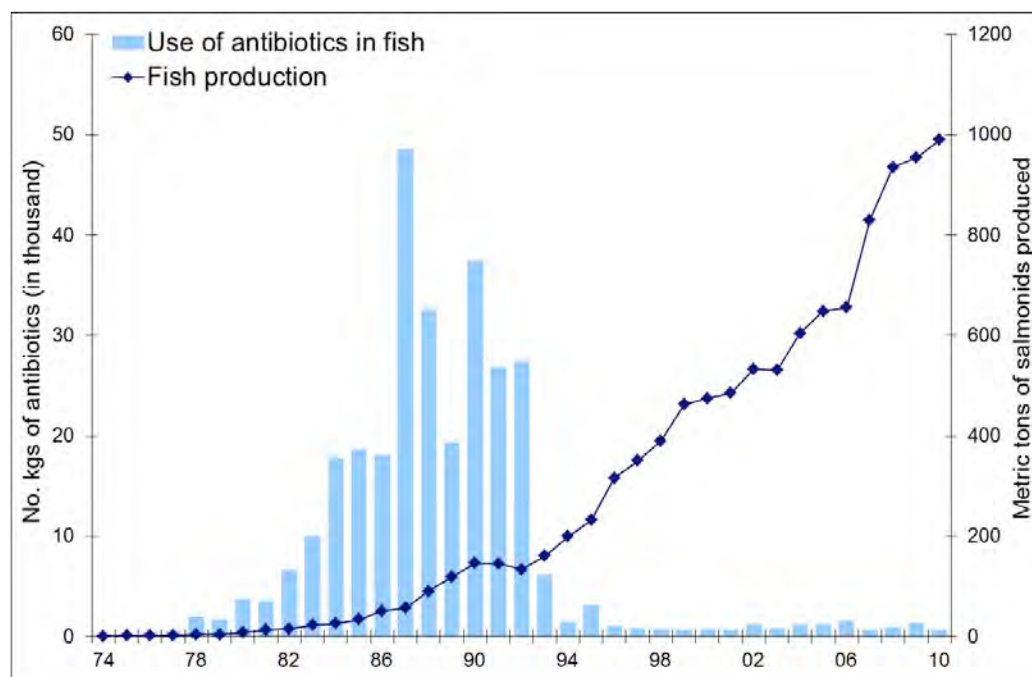
4. Vaccination of farmed fish

4.1. Historical background

The first vaccination trials in fish were conducted by American scientists prior to the Second World War. In Norway, vaccination was first used as a prophylactic measure in 1977 when rainbow trout, and later farmed Atlantic salmon, were vaccinated against vibriosis. Salmonids vaccinated with formalin killed cultures of the relevant *Vibrio anguillarum* serotypes gained good protection against the disease. The vaccine was administered by intraperitoneal injection or by immersion.

During the 1980s, the use of vaccines in the fish farming industry increased dramatically. A few years earlier, a detrimental condition originally known as Hitrasylke, arose in farmed fish. The disease was initially treated with antibiotics and chemotherapeutics. In 1987 the consumption of antibacterial agents in fish farming reached 48.5 tonnes. These high levels were not desirable, so effective vaccines and other preventative measures were a requirement for the growth and development of the entire industry. The disease was later termed cold water vibriosis, as it turned out that it was an infection caused by *Vibrio salmonicida*, and prophylactic vaccination was initiated.

Increased knowledge about adjuvants formed an important part of the foundation for the development of fish vaccines which over the following decade led to a reduction in the use of antibiotics from nearly 50 tonnes to less than one tonne. During the same period, the production of salmon increased enormously.



Consume of antibiotics in Norwegian aquaculture (measured in thousand kgs) and the production of farmed fish (tons).

Today, vaccination is the single most important measure for preventing infectious bacterial diseases in farmed fish. Every year about 250 million fish are vaccinated in Norway.

4.2. Fish diseases where vaccines are used

4.2.1. *Vibriosis*

Vibriosis caused by *Listonella anguillarum* (previously called *Vibrio anguillarum*) is one of the most serious bacterial diseases in fish farming. The disease occurs in all countries with fish farming and the bacterium can cause disease in a wide range of species including salmonids, sea bass, sea bream, cod, turbot and eel. Among the salmonids, the rainbow trout is especially susceptible for the infection.

Vibriosis vaccines include both immersion and injection vaccines. The vibriosis component is now included in the injectable combination vaccines with added adjuvants.

4.2.2. Cold water vibriosis

Cold water vibriosis was first discovered in northern Norway in 1977, and it was then known as "hemorrhagic syndrome." Two years later, there was high mortality in several fish farms at Hitra, and the disease was then called "Hitra disease." After the causal factors were identified, the disease was renamed cold water vibriosis.

As the name indicates, the disease occurs particularly at low temperatures. The infection mainly causes morbidity and mortality of Atlantic salmon. The disease can also occur in rainbow trout and cod, but mortality is moderate or low. These species can be healthy carriers and therefore provide a reservoir for the bacteria. The disease has caused major losses and resulted in extensive medication.

4.2.3. Winter ulcers

Some salmon and rainbow trout develop sores when farmed at low temperatures. Winter ulcers is a descriptive term for this condition based on clinical manifestation and the time of appearance. The occurrence of winter ulcers varies from farm to farm.

Mortality of winter ulcers is generally moderate to low, but there are also reports of cases with high mortality. The importance of the disease for the aquaculture industry has primarily been related to decreased quality as a result of sores, or scars resulting from sores. This reduction of quality of salmon as a result of winter ulcers can therefore lead to great losses in some farms. The condition also poses a welfare issue as it causes osmotic problems in the fish

4.2.4. Furunculosis

Classical furunculosis is an important disease of salmonids in both wild populations and in aquaculture. The disease occurs in most fish farming areas in the Northern hemisphere. Furunculosis causes disease in salmonids in both freshwater and seawater. In 1966, the disease led to mortality in one of Norway's best salmon rivers, Numedalslågen. The waters were monitored carefully after this outbreak and it took 13 years before the disease disappeared.

In Norway, furunculosis was first diagnosed in freshwater farms for rainbow trout in 1964. In 1985, the disease was detected in farmed fish as a result of the importation of infected salmon smolt. Since then, furunculosis has been considered enzootic in Norwegian fish farms. The disease occurs in the seawater phase, with the largest losses occurring in the summer months.

The first furunculosis vaccine was introduced in Norway in 1989. Since then, most of the farmed fish have been vaccinated against furunculosis. Although the infection pressure has been reduced, it is important to maintain the routine vaccination program.

4.2.5. *Infectious pancreatic necrosis – IPN*

IPN is caused by a birnavirus and was first detected in the fry of salmonids in freshwater. Over time the viral disease has been diagnosed in various species of salmon in both freshwater and seawater. IPN also occurs in marine species such as halibut, turbot, cod and eel, and these species may be significant as healthy carriers.

While the IPN had the greatest impact among fry, the disease has in recent years led to high mortality of smolts following release into the sea. It is also possible that the infection may have an immunosuppressive effect on B cells.

The commercially available vaccines against IPN are all inactivated whole cell or subunit vaccines containing surface proteins from viruses. Both the conventional and the recombinant IPN vaccines are included in the combination vaccines with oil adjuvant administered by intraperitoneal injection. The purpose of immunization against IPN is to reduce the losses of smolts following release. No vaccination should therefore take place before smoltification so that immunity is at its highest when the fish is transferred to seawater.

4.2.6. *Infectious salmon anaemia virus – ISA (Infeksiøs lakseanemi – ILA)*

Infectious salmon anaemia (ISA) has had and still has great importance for Norwegian fish farming for several reasons. The disease led to significant losses in many Norwegian fish farms, both because of increased mortality and because of the imposition of slaughter of the fish in infected farms. Internationally, ILA was considered an exotic disease, which made it necessary to take effective measures for combating and prevention.

ISA in Atlantic salmon was first discovered in 1984. The disease is now enzootic in Norway. ISA has also led to outbreaks in other fish farming countries including Scotland, Canada, the Faroe Islands and Chile. As the name suggests, the infection causes anaemia and severe bleeding, which can result in a dark-coloured liver. Rainbow trout and a range of marine fish species do not become clinically ill, but can be carriers.

The fish should be protected when it is transferred to seawater. Optimal time for vaccination is therefore at least 8 weeks or 600 degree days before release [4]. In Norway, immune prophylaxis forms an integral part of a scheme aimed at combating and preventing ISA. According to new regulations vaccination is only allowed in areas permitted by the Norwegian Food Safety Authority (Mattilsynet). To achieve the best possible effect it is important that the population is thoroughly vaccinated.

4.2.7. *Pancreas disease - PD*

Pancreas disease (PD) was first described in Norway in 1989. The disease occurred in a plant in Hordaland, and was of limited economic importance for farmed salmon for the first few years. Since 2003 however, the disease has spread northwards, and the losses due to disease have been substantially greater. PD causes significant losses in several regions, and the disease is considered to be one of the most important in Norwegian fish farming.

Clinical disease usually occurs in the first year at sea, but the disease can also occur in larger fish approaching slaughter. Mortality and reduced growth may vary substantially, depending on genetic and environmental factors. The disease is often diagnosed concurrently with other diseases. In farms with both salmon and rainbow trout, PD is found in both species.

The industry and the government have taken several preventive measures to limit the losses caused by PD. These include movement restrictions, synchronized slaughter and vaccination.

4.3. Welfare consequences of fish vaccinations

Although vaccination of fish is conducted to protect the animals against disease and thus improve animal welfare, the procedure may at the same time lead to a number of negative welfare consequences for the animals involved. The most important of these will be briefly described below.

4.3.1. *Immune reactions and adhesions*

Immune reactions and adhesions, both between organs and between organs and the abdominal wall, are very common side effects that are clearly linked to tissue irritation and inflammation caused by the oil adjuvant and antigen mix. The vaccine depot that is achieved when using oil-based vaccines stimulates the immune system for a prolonged period. This may again cause inflammation and subsequent adhesions. If the adhesions are severe, they may reduce the function of the gastrointestinal and reproductive organs.

4.3.2. *Melanin deposition*

Vaccination leads to a normal immune response that also involves influx of melanomacrophages and other white blood cells. These cells can release the brown pigment melanin on the peritoneum, or in (or on the surface of) internal organs. Normally the pigmentation is removed at the slaughterhouse, but in severe cases it can be left in the abdominal wall and may be an important cause of quality

downgrading. A correlation between adhesions in the abdominal cavity and melanin deposition is also observed [8].

It has been assumed that the pigmentation itself does not pose a welfare problem for the fish, as melanin is the result of a normal immune reaction. However, if melanin depositions occur due to trauma, infection, or irritation with subsequent inflammation, necrotic tissue and muscle degeneration, the damage is likely to lead to discomfort for the fish, without the vaccine necessarily being the cause [6].

4.3.3. Reduced growth

Reduced growth following vaccination can have both short and long term effects. The reduction seen immediately after vaccination is brief. Vaccinated fish may exhibit compensatory growth, so that they are the same size as unvaccinated fish when released into seawater. Salmon show reduced growth after being vaccinated, but there are also other reasons for this short-term growth reduction, including stress, pain, poor energy consumption and energy spent on generating the immune reaction [6].

When it comes to the long-term effects on growth, different studies have come to different conclusions. Some have demonstrated reduced growth in seawater in vaccinated fish compared with unvaccinated animals, while others have failed to find any difference. There are also reports of vaccinated fish showing better growth than unvaccinated fish [6].

Several factors may cause growth reduction. In severe cases of adhesions, the physical damage may compromise the ability of the fish to eat, digest its food and transport ingesta through the gastrointestinal tract. Organ damage may impair function and immune reactions and healing. Discomfort may alter metabolism and the allocation of available energy. These can cause changes in behaviour or appetite with reduced feed intake. Under normal conditions with moderate adhesions, vaccination is not expected to cause major physical damage to the digestive system [6].

4.3.4. Skeletal deformations

There are a number of factors that may cause spinal deformities in farmed salmon, including small smolt, rapid growth, the wrong time of vaccination, low phosphorous content, foreign matter and high incubation temperature.

Skeletal deformations are one of the most significant factors for economic losses in salmon farming. Although hard to quantify, one study showed an average loss of 7.5% reduction in classification due to deformations. The economic loss was estimated to be 2-3 NOK/kg slaughtered salmon [6].

5. Development of a fish vaccine

5.1. Laws and regulations

Legislation on the control and administration of vaccines varies greatly from country to country, depending partly on the country's size and customs. In many countries, there is one agency for human and veterinary medicines, that controls both immunological and pharmaceutical preparations. In other countries, the agency for veterinary medicines is a separate entity.

Vaccine development and validation in Norway is regulated by national and European legislation (Guidelines and Monographs). A Norwegian vaccine producer must have a valid manufacturing permit (*tilvirkertilatelse*) from the Norwegian Medicines Agency. Authorization to produce a pharmaceutical is based on extensive documentation which demonstrates that production was in accordance with the European Standard (Good Manufacturing Practice). Permits are given for a limited time period and all manufacturers are subject to inspection. Manufacturers wishing to sell vaccines on the Norwegian market must also apply for a marketing permit. To obtain this from the Norwegian Medicine Agency, the producer needs to document pharmaceutical quality, safety and therapeutic effect.

Foreign producers within the European Economic Area (EEA) are required to apply for similar approval from their home country. The EEA inspectorates have formal collaboration and free access to all inspection reports. Manufacturers in a third country outside the EEA are required to be inspected as regards GMP and must be approved by the Medicines Agency from an EEA country. Norwegian authorities demand information on production routines and control measures from foreign producers when new marketing authorisations are evaluated. If considered necessary, Norwegian Medicine Agency authorities (*legemiddelmyndigheten*) can demand to inspect the production facilities of a third country producer.

More detailed information can be collected from

http://www.legemiddelverket.no/templates/InterPage_30299.aspx

5.2. From the laboratory to the ocean – the need for fish in the different phases of vaccine development

Developing and testing a new fish vaccine is an extensive operation which can be divided into the following steps:

1. Phase 1 – Feasibility (laboratory studies)
 - a. Virulence testing
 - b. Challenge models
 - c. Cross protection studies
 - d. Dose titration studies



2. Phase 2 – Development (laboratory/field studies)

- a. Field studies
- b. Onset of immunity
- c. Duration of immunity (DOI)
- d. Safety
- e. Potency
- f. Stability
- g. Dose-finding

3. Phase 3 - Documentation (laboratory/field trials)

- a. Safety
- b. Efficacy

4. Phase 4 - Post licensing (post marketing studies)

- a. Field studies
- b. Batch release (safety and potency)
- c. Stability testing (safety and potency)

Estimating the exact number of fish that are needed in the various phases is very difficult and relies on a number of factors that include the complexity of the vaccine, trial success and the agents concerned. The following presentation is an example of the number of fish required in each phase:

5.2.1. Phase 1 – Feasibility (lab studies)

Phase 1 in the development of a new fish vaccine is called the feasibility phase. It is carried out in the laboratory and encompasses several smaller scale studies:

Virulence testing by exposing fish to the disease agent

- 800 fish per study (4 strains × 2 administration methods × 50 fish × 2 replicates)

Development of challenge models

- 800 fish per study (4 administration methods × 2 groups × 50 fish × 2 replicates)

Cross protection studies in target species

- 2000 fish per study (2 groups × 100 fish × 5 challenge strains × 2 replicates)

Dose titration studies including challenge

- 2000 fish per study (5 doses × 100 fish × 2 groups × 2 replicates)

The number of fish used in phase 1 is dependent upon the success rate. Some vaccines are easy to develop, while other studies may go on for years.



5.2.2. Phase 2 – Development (lab/field studies)

Duration of protection

- Mini cage trials suitable for field safety documentation
- Commercial scale trials useful for monitoring growth of vaccinated fish
- Field trials are not suitable for documentation of duration of protection
 - o Outbreak of disease rarely occurs
 - o Antibody analysis?
- Field duration of protection studies have been replaced by laboratory duration of protection studies – the number of animals has been reduced

Test	Guidelines	Challenge time	No. of fish (total)	Observation period
Efficacy	Ph. Eur.			21 days after the first death
Monovalent		6 months	400	
Hexavalent		6 months	2400	
		12 months	2400	

5.2.3. Phase 3 - Documentation (lab/field trials)

The next step is to document the safety and efficacy of the vaccine. This is done both in the laboratory and in the field.

Documentation of safety- laboratory

3 batches are tested

Test	Guidelines	No. of fish per batch	Total no. of fish	Observation period
Double dose safety	Ph. Eur.	50	150 (vaccinated) + 50 (controls)	21 days
Field trials	Ph. Eur.	Not defined	Not defined	Until slaughter

Documentation of efficacy- laboratory

Test	Guidelines	No. of fish per batch and antigen	Total no. of fish	Observation period
Efficacy	Ph. Eur.	100		21 days after the first death
Monovalent			800	
Hexavalent			4000	



Documentation of efficacy - field

- Trial in mini cages
 - o Two replicate cages
 - o 1000 – 3000 fish per cage
 - o 6 – 8 groups per cage
 - o Two premises run in parallel

5.2.4. Phase 4 – Post licensing (Post marketing studies)

Batch testing

Every batch must be tested for potency and safety according to the European Pharmacopoeia.

- Safety
 - o 10 fish injected with a double dose per batch
- Potency
 - o Minimum 30 fish vaccinated and challenge-tested per antigen per batch
 - 70 fish for monovalent vaccines
 - 420 fish for hexavalent vaccines

Documentation of efficacy - field

- Trial in production cages
 - o One cage
 - o 100 000 fish per cage
 - o 10 – 50% of the fish sent to market

It has been estimated that, during development and documentation, up to 20 000 fish per product are used. In the batch release testing of the final products, up to 15 000 fish are used, while clinical field trials on a commercial scale with licensed products may use several hundred thousand fish per study.

6. Areas with potential for improvement

6.1. Replacement of challenge tests with antibody tests

The procedure most frequently used to test fish vaccines today is a challenge test. This entails vaccinating the fish and letting immunity develop before infecting the fish with the agent(s) the vaccine is supposed to protect against. The number of survivors is then compared to a non-vaccinated population to test vaccine

efficiency. This is both time-consuming and expensive, and requires a lot of test animals.

A recent experiment, carried out in Norway on Atlantic salmon by Romstad et al [7], shows promising results in this regard. The study set out to assess the antibody response development for *Aeromonas salmonicida* vaccines and the correlation between antibody response and protection in cohabitation challenge. The authors found that fish vaccinated with a full antigen dose had a significant increase in antibody response after 252 day degrees, and a correlation of 0.94 was found between the antibody response and protection after 500 day degrees.

These findings indicate that an immunogenicity test can discriminate between vaccines of different antigen content. Following further validation this method therefore has the potential to replace challenge tests in vaccine tests.

6.2. Response Surface Pathway (RSP)

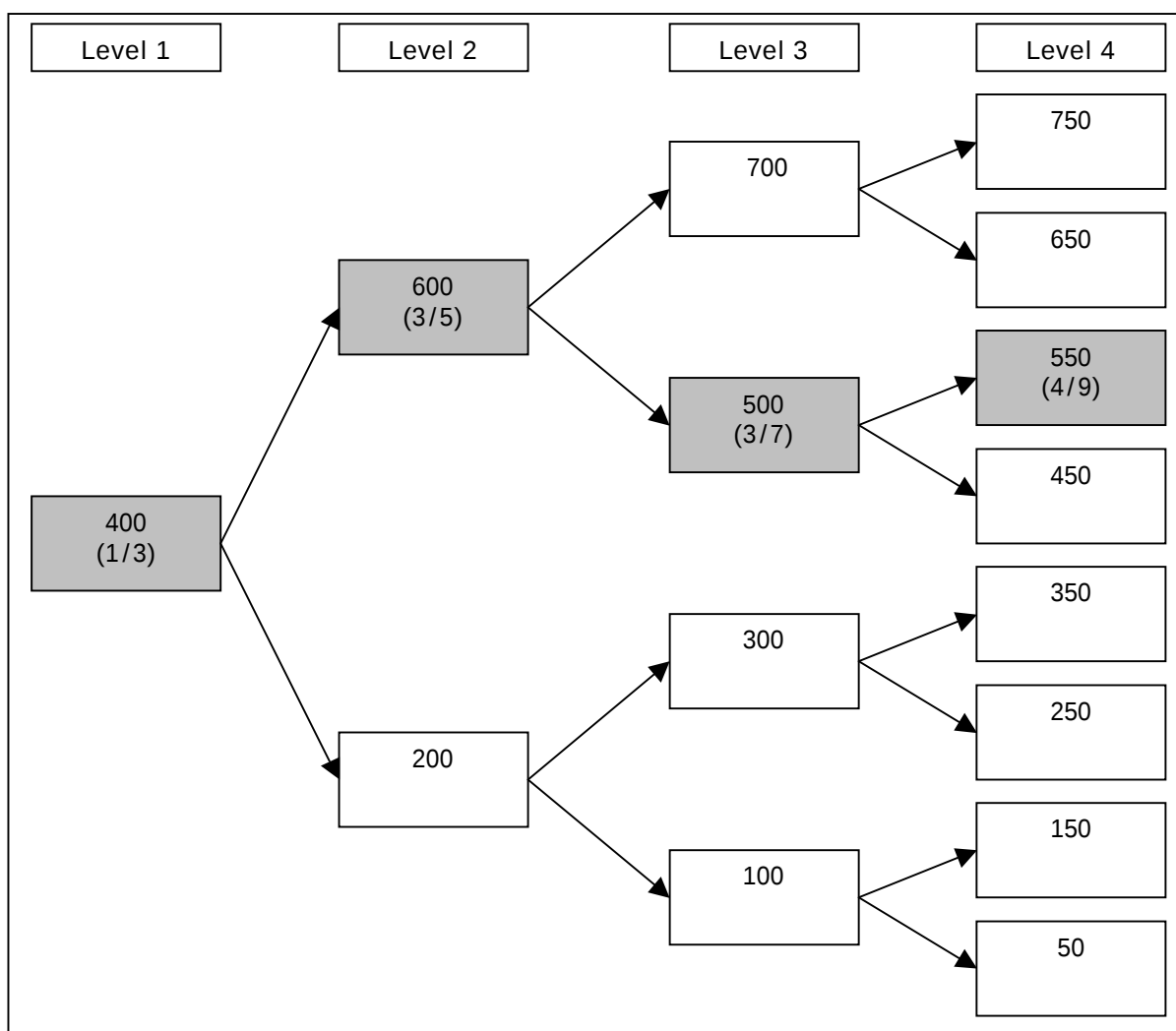
RSP was originally a technique developed in connection with LD₅₀ testing of chemicals and pharmaceuticals. The principles in RSP are, however, also applicable to dose titration studies during phase 1 of vaccine development to determine the optimum level of a vaccine agent.

RSP is a method of study design where dose levels in a toxicity trial are adjusted to minimise the number of animals that are exposed to toxic levels of a substance. The trial is split into levels with different dose rates, and uses low numbers of animals at each level. The dose rate used at each level is determined by the outcome (mortality) at the previous level. Dose rates are adjusted up or down depending on the animals' response at the previous level (numbers of dead animals in brackets).

Dose rates are calculated by the following formula:

If it has been determined that a vaccine concentration of e.g. 700 kills 100% of the test subjects, while 100 kills 0%, the initial dose rate would be:

$D_1 = (100 + 700)/2 = 400$ [8]. To make the adjustment more sensitive in level 2, level two is calculated by: $D_2 = D_1 \pm (D_1/2)$. In the example below, this would mean 600 or 200 depending on the number of dead animals. For level 3 and 4 the adjustments are made even more sensitive: $D_3 = D_1 \pm (D_1/2) \pm (D_1/4)$ and $D_4 = D_1 \pm (D_1/2) \pm (D_1/4) \pm (D_1/6)$.



Response Surface Pathway design has the potential to reduce the number of laboratory animals by about 70% without loss in information.

RSP has several advantages [8]:

- It can be used together with other designs
- It increases the information from a given number of laboratory animals
- It reduces the number of laboratory animals without loss of information
- The protocol is not predetermined but depends upon the outcome of the previous investigation
- It allows a stochastic approach to investigations

6.3. Humane endpoints

An alternative to counting the number of dead fish in a vaccine trial is to use humane endpoints. This is a term referring to an animal welfare acceptable condition where a test animal, in this context a fish, is no longer exposed to

further strain and is excluded from the trial. Humane endpoint can either apply to individuals in a group, the entire group of the entire trial [10].

The definition of the humane endpoint relies on the type of trial, parameters to be measured and knowledge about how the experimental settings affect the fish. There is often overlap between a humane endpoint and welfare indicators, as the former is generally based on the latter. Trials on mammals usually have clear and well-defined endpoints, while endpoints in fish trials often are more general. It is therefore an apparent need for precise and trial specific humane endpoints in vaccine development and validation.

6.4. Fundamental factors

There are certain fundamental factors which need to be in place in order to secure refinement and reduction in animal numbers, whether terrestrial or aquatic. These factors are listed below. These factors apply to all phases of vaccine development and testing, regardless of vaccine type.

Factors to consider in relation to refinement and reduction [9].

Materials and equipment	Use of appropriate, well-maintained and (where relevant) sterile equipment. Careful preparation, maintenance and storage of materials, and consideration of their nature (e.g. irritancy, tissue compatibility, sterility, temperature) when administered.
Criteria for selection of animals	Selection of an appropriate species and strain of animals with consideration of other factors such as age, weight and sex. Use of a consistent source of animals with good health status.
Animal husbandry and care	Animal housing and care that takes into account the physical and behavioural needs of the animals as well as the need to be able to monitor them without too much disturbance. Use of gentle handling and restraint procedures.
Numbers of animals and statistical design	Application of appropriate experimental and statistical design with justification of the numbers of animals. Timing of the vaccine challenge to facilitate monitoring (in relation to the animals, time, budget and staff availability).
Administration of substances	Use of the most refined methods including: - an appropriate gauge needle (i.e. the smallest gauge appropriate to the species, route and substance administered); - selection of the least invasive route likely to cause the least trauma and pain to the animals; - selection of appropriate and least harmful site(s) for administration and suitable preparation of the site to facilitate accurate administration the first time; - use of aseptic technique; - exploration of opportunities to reduce the volume administered.



Humane endpoints	Description and implementation of humane endpoints to minimise the degree and duration of suffering.
Monitoring animals	Careful, regular and timely monitoring of animals for adverse effects including those associated with the administration procedure itself. Use of anaesthetic and analgesics to reduce pain.
Staff	Sufficient, appropriately trained and competent staff who can implement all of the above.

7. Recommendations

The 3Rs (Replacement, Reduction and Replacement) of Russell & Burch originated from a project initiated in 1954 by the Universities Federation of Animal Welfare (UFAW). They defined the 3Rs as:

- Replacement: methods which permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals
- Reduction: methods for obtaining comparable levels of information from the use of fewer animals in scientific procedures, or for obtaining more information from the same number of animals
- Refinement: methods which alleviate or minimise potential pain, suffering or distress, and which enhance animal well-being

The recommendations in the report “Three Rs approaches in the production and quality control of fish vaccines” [2] are generally supported. The report emphasised the need for more research. However, we believe that a number of recommendations can be made already, based upon current knowledge. These involve, among other things, the substitution of “recommendations” with “requirements”.

Our recommendations are summarised below:

7.1. Replacement

Development of a fish vaccine requires live fish which are challenged for a number of reasons, including virulence testing of micro-organisms and testing of protective immunity afforded by experimental vaccines.

Batch potency testing based on quantification of antigens, where the batch results are compared with the results of a reference vaccine, is a feasible alternative. For the time being there does not seem to be scientific and technical basis for this replacement. However, by research and development in scientific institutions and in the industry replacement of some in vivo tests by in vitro tests is a goal that can be reached.

1. In the documentation phase, and especially in the post-licensing phase, *in vivo* tests can be substituted by *in vitro* tests. The content of the antigens responsible for protective immunity can be measured in the laboratory, for instance by an ELISA test.
2. The vaccine industry should be encouraged to put more emphasis on replacement in the development of vaccines for aquatic animals.
3. The regulatory authorities should include such requirements in their communication with the industry when issuing a licence.
4. Universities and other scientific institutions should plan projects with the replacement of experimental fish as an aim.
5. Research Councils should give priority to projects providing a scientific and technical basis for replacement.
6. Economic support for replacement methods should be increased by political means.

7.2. Reduction

There is a potential for reduction of the number of fish used during the various phases of development, documentation and post-licensing. The number of fish used in the different phases should be high enough to give reliable results. However, if statistical methods are applied when the number of experimental fish is calculated, there is a potential for reduction.

1. RSP study design, or similar methods, should be used to reduce the number of fish used in vaccine trials, particularly in the development phase.
2. When planning challenge experiments or other tests associated with pain, the statistical method should be chosen with care and justified.
3. There seems to be a discrepancy between the European Pharmacopoeia monograph and EU guidelines. This discrepancy should be removed.
4. A reduction in the genetic variation within fish breeds would reduce the number of fish required for vaccine development and validation.
5. Although the use of fish in safety testing does not appear to be a topical welfare issue, the use of fish for this purpose should be well justified.

7.3. Refinement

The potency test used as a basis for batch release of fish vaccines is based on vaccination followed by a challenge test. For most other veterinary vaccines, potency testing is performed using in vitro tests or by serological tests.

There are several scientific papers on the immune response after vaccination of salmonid fish. A recent publication demonstrates a strong correlation between antibody response and survival rates in Atlantic salmon in connection with furunculosis [7].

There is no similar data for other antigens so far. This means that challenge tests should still be used for multivalent vaccines. However, there is reason to believe that a correlation exists for other antigens as well. Lower correlation coefficients may be acceptable as a basis for serological potency tests. Tests based on serology will reduce pain in experimental fish significantly, compared to standard challenge tests.

The publication mentioned above [7], together with other studies over the last twenty years, provides in our opinion sufficient basis for a change in the requirements for potency testing of vaccines against furunculosis.

1. The fish vaccine industry should be requested to validate the *in vitro* test for potency testing of furunculosis vaccine.
2. The industry should also be requested to undertake similar studies for the other components of fish vaccines.
3. Vaccine producers should be requested to join efforts to collect all existing data to adequately document correlations between titre and protection in challenge tests for all agents. This would lead to a more rapid replacement of batch potency with antibody measurements.
4. Well-defined and trial specific humane endpoints in vaccine development and validation need to be developed and used.

8. Acknowledgements

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Virus-Serum-Toxin Act
21 USC 151-159 et. seq.

CHAPTER 5. VIRUSES, SERUMS, TOXINS, ANTITOXINS, AND ANALOGOUS PRODUCTS

151. Preparation and sale of worthless or harmful products for domestic products for animals prohibited, preparation to be in compliance with rules at licensed establishments

152. Importation regulated and prohibited

153. Inspection of imports; denial or entry and destruction

154. Regulations for preparation and sale; licenses

154a. Special licenses for special circumstances; expedited procedure; conditions; exemptions; criteria

155. Permits for importation

156. Licenses condition on permitting inspection; suspension of licenses

157. Same; inspection daytime or nighttime

158. Offenses; punishment

159. Corporations; commerce; trade

(37 Stat. 832-833; as amended December 23, 1985, Pub. L. 99-198, 99 Stat.1654-1655; 21 U.S.C. 151-159).

151. Preparation and sale of worthless or harmful products for domestic animals prohibited; preparation to be in compliance with rules at licensed establishments.

It shall be unlawful for any person, firm or corporation to prepare, sell, barter, or exchange in the District of Columbia, or in the Territories or in any place under the jurisdiction of the United States, or to ship or deliver for shipment in or from the United States, the District of Columbia, any territory of the United States, or any place under the jurisdiction of the United States, any worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous product intended for use in the treatment of domestic animals, and no person, firm, or corporation shall prepare, sell, barter, exchange, or ship as aforesaid any virus, serum, toxin, or analogous product manufactured within the United States and intended for use in the treatment of domestic animals, unless and until the said virus, serum, toxin, or analogous product shall have been prepared, under and in compliance with regulations prescribed by the Secretary of Agriculture, at an establishment holding an unsuspended and unrevoked license issued by the Secretary of Agriculture as herein after authorized.

152. Importation regulated and prohibited.

The importation into the United States, without a permit from the Secretary of Agriculture, of any virus, serum, toxin, or analogous product for use in the treatment of domestic animals, and the importation of any worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous product for use in the treatment of domestic animals, are hereby prohibited.

153. Inspection of imports; denial of entry and destruction.

The Secretary of Agriculture is hereby authorized to cause the Bureau of Animal Industry to examine and inspect all viruses, serums, toxins, and analogous products, for use in the treatment of domestic animals, which are being imported or offered for importation into the United States, to determine whether such viruses, serums, toxins, and analogous products are worthless contaminated, dangerous, or harmful, and if it shall appear that any such virus, serum, toxin, or analogous product, for use in the treatment of domestic animals, is worthless, contaminated, dangerous, or harmful, the same shall be denied entry and shall be destroyed or returned at the expense of the owner or importer.

154. Regulations for preparation and sale; licenses.

The Secretary of Agriculture is hereby authorized to make and promulgate from time to time such rules and regulations as may be necessary to prevent the preparation, sale, barter, exchange, or shipment as aforesaid of any worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous product for use in the treatment of domestic animals, or otherwise to carry out this paragraph, and to issue, suspend, and revoke licenses for the maintenance of establishments for the preparation of viruses, serums, toxins, and analogous products, for use in the treatment of domestic animals, intended for sale, barter, exchange, or shipment as aforesaid.

154a. Special licenses for special circumstances; expedited procedure; conditions; exemptions; criteria.

In order to meet an emergency condition, limited market or local situation, or other special circumstance (including production solely for intrastate use under a State program), the Secretary may issue a special license under an expedited procedure on such conditions as are necessary to assure purity, safety, and a reasonable expectation of efficacy. The Secretary shall exempt by regulation from the requirement of preparation pursuant to an unsuspended and unrevoked license any virus, serum, toxin, or analogous product prepared by any person, firm, or corporation--

- (1) solely for administration to animals of such person, firm, or corporation;
- (2) solely for administration to animals under a veterinarian-client-patient relationship in the course of the State licensed professional practice of veterinary medicine by such person, firm, or corporation; or
- (3) solely for distribution within the State of production pursuant to a license granted by such State under a program determined by the Secretary to meet the criteria under which the State--
 - (A) may license virus, serum, toxin, and analogous products and establishments that produce such products;
 - (B) may review the purity, safety, potency, and efficacy of such products prior to licensure;
 - (C) may review product test results to assure compliance with applicable standards for purity, safety, and potency prior to release to the market;
 - (D) may deal effectively with violations of State law regulating virus, serum, toxin, and analogous products; and
 - (E) exercises the authority referred to in subclauses (A) through (D) consistent with the

intent of this paragraph of prohibiting the preparation, sale, barter, exchange, or shipment of worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous products.

155. Permits for importation.

The Secretary of Agriculture is hereby authorized to issue permits for the importation into the United States of viruses, serums, toxins, and analogous products, for use in the treatment of domestic animals, which are not worthless, contaminated, dangerous, or harmful.

156. Licenses conditioned on permitting inspection; suspension of licenses.

All licenses issued under authority of this chapter to establishments where such viruses, serums, toxins, or analogous products are prepared for sale, barter, exchange, or shipment as aforesaid, shall be issued on condition that the licensee shall permit the inspection of such establishments and of such products and their preparation; and the Secretary of Agriculture may suspend or revoke any permit or license issued under authority of said chapter, after opportunity for hearing has been granted the licensee or importer, when the Secretary of Agriculture is satisfied that such license or permit is being used to facilitate or effect the preparation, sale, barter, exchange, or shipment as aforesaid, or the importation into the United States of any worthless, contaminated, dangerous, or harmful virus, serum, toxin, or analogous product for use in the treatment of domestic animals.

157. Same; inspection daytime or nighttime.

Any officer, agent, or employee of the Department of Agriculture duly authorized by the Secretary of Agriculture for the purpose may, at any hour during the daytime or nighttime, enter and inspect any establishment where any virus, serum, toxin, or analogous product for use in the treatment of domestic animals is prepared for sale, barter, exchange, or shipment as aforesaid.

158. Offenses; punishment.

Any person, firm, or corporation who shall violate any of the provisions of this chapter shall be deemed guilty of a misdemeanor, and shall, upon conviction, be punished by a fine of not exceeding \$1,000 or by imprisonment not exceeding one year, or by both such fine and imprisonment, in the discretion of the court.

159. Enforcement, penalties applicable. Congressional findings.

The procedures on sections 672, 673, and 674 of this title (relating to detentions, seizures and condemnations, and injunctions, respectively) shall apply to the enforcement of this chapter with respect to any product prepared, sold, bartered, exchanged, or shipped in violation of this chapter or a regulation promulgated under this chapter. The provisions (including penalties) of section chapter 675 of this title shall apply to the performance of official duties under this paragraph. Congress finds that (1) the products and activities that are regulated under this chapter are either in interstate or foreign commerce or substantially affect such commerce or the free flow thereof and (ii) regulations of the products and activities as provided in this chapter is necessary to prevent and eliminate burdens on such commerce and to effectively regulate such commerce.

This section is effective on December 23, 1985, except that:

(1) Subject to subparagraphs (2) through (4), in the case of a person, firm or corporation preparing, selling, bartering, exchanging, or shipping a virus, serum, toxin, or analogous product during the 12 month period ending on the date of enactment of this Act solely for intrastate commerce or for exportation, such product shall not after such date of enactment, as a result of its not having been licensed or produced in a licensed establishment, be considered in violation of the eighth paragraph of the matter under the heading BUREAU OF ANIMAL INDUSTRY" of the Act entitled The Virus- Serum-Toxin Act.

An Act making appropriations for the Department of Agriculture for fiscal year ending June thirtieth, nineteen hundred and fourteen", approved March 14, 1913 (as amended by this section), until the 1st day of the 49th month following the date of enactment of this Act.

(2) The exemption granted by subparagraph (1) may be extended by the Secretary of Agriculture for a period up to 12 months in an individual case on a showing by a person, firm, or corporation of good cause and a good faith effort to comply with such eighth paragraph with due diligence.

(3) The exemption granted by subparagraph (1) must be claimed by the person, firm, or corporation preparing such product by the 1st day of the 13th month following the date of enactment of this Act, in the form and manner prescribed by the Secretary, unless the Secretary grants an extension of the time to claim such exemption in an individual case for good cause shown.

(4) On the issuance by the Secretary of a license to such person, firm, or corporation for such product prior to the 1st day of the 49th month following the date of enactment of this Act, or the end of an extension of the exemption granted by the Secretary, the exemption granted by subparagraph (1) shall terminate with respect to such product.

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(i) Any adverse reactions shall be recorded.

(ii) The dosage of reference administered to each animal shall be in accordance with label directions. Label directions may indicate a single dosage regardless of weight, in which case the animals in the study shall be at or near the maximum weight for neonates of the species.

(4) After 24 hours, blood samples shall be taken from each animal.

(5) Pretreatment and post treatment serum IgG concentrations shall be concurrently determined for each animal using a radial immunodiffusion (RID) method acceptable to APHIS and described in the filed Outline of Production for the product.

(6) Concurrently, using the same method, five IgG measurements shall be made on an IgG Species Standard supplied or approved by APHIS. The IgG Species Standard shall be a preparation that contains IgG specific for the species in question at a concentration acceptable to APHIS.

(7) For an IgG Reference Product to be satisfactory, all animals used to qualify the reference must remain free of unfavorable product-related reactions and at least 90 percent of the paired serum samples must reflect an increase in IgG concentration (posttreatment minus pretreatment concentration) equal to or greater than the IgG concentration of the IgG Species Standard.

(b) *Antibody functionality.* Prior to licensure, the prospective licensee shall perform a neutralization study, or another type of study acceptable to APHIS, to demonstrate functionality of product antibody.

(c) *Potency.* Bulk or final container samples of completed product from each serial shall be tested for IgG content as provided in this paragraph. Samples of the test serial and of an IgG Reference Product established in accordance with paragraph (a) of this section shall be concurrently tested for IgG content by the RID method referred to in paragraph (a)(5) of this section. Five IgG measurements shall be made on each. If the IgG level per dose of the test serial does not meet or exceed that of the reference, one complete retest, involving five IgG meas-

urements on both the reference and two samples of the test serial, may be conducted. If, upon retest, the average IgG level per dose of the two samples of the test serial does not meet or exceed that of the reference, or if a retest is not conducted, the serial is unsatisfactory.

[61 FR 51777, Oct. 4, 1996]

PART 114—PRODUCTION REQUIREMENTS FOR BIOLOGICAL PRODUCTS

Sec.

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- 114.17 Rebottling of biological products.
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AUTHORITY: 21 U.S.C. 151-159; 7 CFR 2.22, 2.80, and 371.4.

SOURCE: 39 FR 16369, May 10, 1974, unless otherwise noted.

§114.1 Applicability.

Unless exempted by regulation or otherwise authorized by the Administrator, all biological products prepared, sold, bartered or exchanged, shipped or delivered for shipment in or from the United States, the District of Columbia, any Territory of the United States, or any place under the jurisdiction of the United States shall be prepared in accordance with the regulations in this part. The licensee or permittee shall adopt and enforce all necessary measures and shall comply with all directions the Administrator prescribes for carrying out such regulations.

[52 FR 11026, Apr. 7, 1987, as amended at 56 FR 66784, Dec. 26, 1991]

in legends filed in accordance with 9 CFR part 108. A description of each State-licensed product must be filed with the Animal and Plant Health Inspection Service as part of the blueprint legends and must be sufficient for Animal and Plant Health Inspection Service to determine any risk to the production of other products in the licensed establishment and to determine that adequate procedures are followed to prevent contamination during production.

(3) Records in such establishments must be maintained in accordance with §§116.1 and 116.2 of this subchapter and shall include all products licensed by the State or USDA.

(4) Reports prescribed in § 116.5 of this subchapter for USDA-licensed establishments shall be submitted for all veterinary biological products in the establishment.

(5) Under the following conditions, an autogenous biologic may be produced in a USDA-licensed establishment under either a State or U.S. Veterinary Biological Product License:

(i) When a culture of microorganisms, isolated from a herd in a State, is received at a USDA-licensed establishment that is in the same State but that holds both a State and a U.S. Veterinary Biological Products License for autogenous biologics, the isolate shall be designated by the licensee for use in the production of an autogenous biological product under either the State product license, or the U.S. Veterinary Biological Product License: *Provided*, That the isolate meets the requirements of the respective regulatory authority for an autogenous biologic. If, after producing the product pursuant to one license, the licensee elects to produce an autogenous biologic from the same isolate under provisions of the other license, the licensee may do so only with the approval of the other licensing authority.

(ii) The true name of a State-licensed autogenous biologic shall specify the State of licensure; e.g.

“_____Autogenous Bacterin”
(State)
or _____Autogenous Vaccine”.

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(State)

[39 FR 16869, May 10, 1974, as amended at 60 FR 48021, Sept. 21, 1995]

§ 114.3 Separation of establishments.

(a) Each licensed establishment shall be separate and distinct from any other establishment in which a biological product is prepared.

(b) No biological products authorized to be prepared in a licensed establishment shall be prepared in whole or in part by another licensed establishment except as provided in paragraphs (c) and (d) of this section.

(c) When a partially prepared biological product cannot be completed at a licensed establishment due to failure of essential equipment, the Administrator may authorize the use of similar equipment at another licensed establishment: *Provided*, That, such authorization shall be limited to the duration of the emergency and to the phase of production affected by the equipment failure.

(d) Partially prepared products or serials of completed products for further manufacture may be moved from one licensed establishment to another licensed establishment, imported under the provisions of § 104.5, or moved from a licensed establishment for purpose of being exported under conditions prescribed in an Outline of Production filed with Animal and Plant Health Inspection Service. Licensed products or products imported for distribution and sale may be prepared and recommended for final use, for further manufacturing purposes, or both. All serials shall be subject to the requirements for testing and release specified in § 113.5 or § 113.10 and to the requirements for identification specified in § 114.4.

[39 FR 16869, May 10, 1974, as amended at 40 FR 46093, Oct. 6, 1975; 49 FR 45846, Nov. 21, 1984; 56 FR 66784, Dec. 26, 1991]

§ 114.4 Identification of biological products.

Suitable tags or labels of a distinct design shall be used for identifying all ingredients used in the preparation of biological products, all component parts to be combined to form a biological product, all biological products while in the course of preparation and all completed biological products held

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in storage at licensed establishments: *Provided*, That, if such ingredients, components, or biological products are not so identified, they shall be disposed of as provided in § 114.15.

§ 114.5 Micro-organisms used as seed.

Micro-organisms used in the preparation of biological products at licensed establishments shall be free from the causative agents of other diseases or conditions. A complete record of such micro-organisms shall be kept currently correct and a list submitted to Animal and Plant Health Inspection Service upon request of the Administrator.

(Approved by the Office of Management and Budget under control number 0579-0059)

[39 FR 16869, May 10, 1974, as amended at 48 FR 57473, Dec. 30, 1983; 56 FR 66784, Dec. 26, 1991]

§ 114.6 Mixing biological products.

Each biological product, when in liquid form, shall be mixed thoroughly in a single container. During bottling operations, the product shall be constantly mixed sufficient to maintain physical uniformity of the entire fill. A serial number, with any other markings that may be necessary for ready identification of the serial, shall be applied to identify it with the records of preparation and labeling.

§ 114.7 Personnel at licensed establishments.

(a) Each licensee shall designate a person(s) to make all official contacts with Animal and Plant Health Inspection Service on matters pertaining to the preparation of biological products under the Virus-Serum-Toxin Act. The licensee shall file three copies of biographical summary with Animal and Plant Health Inspection Service for such designated person and for each person responsible for any phase of preparation of a biological product.

(b) All personnel employed in the preparation of biological products at a licensed establishment shall be competent in good laboratory techniques through education or training, or both, so as to consistently prepare high quality products.

(c) All biological products prepared at licensed establishments shall be prepared and handled with due sanitary precautions. Good sanitary measures shall be practiced at all times by all personnel involved in such preparation and handling of biological products.

(1) The clothing worn by persons while preparing biological products shall be clean. All persons, immediately before entering laboratory rooms of a licensed establishment, shall change their outer clothing or effectively cover the same with gowns or other satisfactory clean garments.

(2) Unsanitary practices such as, but not limited to, eating, smoking, or expectorating on the floors or otherwise creating a nuisance in any room, compartment, or place in which biological products are prepared, handled, or stored at licensed establishments are prohibited.

(Approved by the Office of Management and Budget under control number 0579-0013)

[39 FR 16869, May 10, 1974, as amended at 48 FR 57473, Dec. 30, 1983; 56 FR 66784, Dec. 26, 1991]

§ 114.8 Outline of Production required.

An Outline of Production shall be on file with Animal and Plant Health Inspection Service for each licensed biological product or for each biological product authorized to be imported into the United States for Distribution and Sale. Preparation of a biological product in a licensed establishment shall be in accordance with the Outline of Production for such product filed with Animal and Plant Health Inspection Service as provided in this section, but subject to changes as may be required under § 114.8(f).

(a) The Outline of Production shall be prepared as prescribed in § 114.9 and submitted to Animal and Plant Health Inspection Service for filing. When objectionable features, if any, are corrected and no further exceptions are taken by Animal and Plant Health Inspection Service to an Outline of Production for a biological product, such Outline of Production shall be approved for filing.

(b) Each page shall be stamped as filed on the date such action was taken in the bottom right hand corner. Although the filed outline may be re-

ferred to as an approved outline, approval for filing constitutes no endorsement by Animal and Plant Health Inspection Service of such biological product or the methods and procedures used to prepare such biological product.

(c) One copy of the Outline of Production shall be retained by the Animal and Plant Health Inspection Service and one copy returned to the licensee or permittee.

(d) Each licensee shall review each Outline of Production for accuracy and sufficiency not less frequently than once a year. Revisions necessary to bring an Outline of Production into compliance with the regulations shall be submitted to Animal and Plant Health Inspection Service.

(e) When a list of licensed products to be continued in production at a licensed establishment is requested by the Administrator in accordance with § 102.5(d) of this subchapter, the licensee shall supplement the list with information for each product as follows:

(1) The Outline of Production currently being used shall be identified as to the date when last revised and filed with Animal and Plant Health Inspection Service and the date of the last review made by the licensee.

(2) The Outline of Production to be kept in the active file shall be designated. If more than one has been filed for a product, only the Outline of Production currently being used shall be included.

(f) The Administrator may, upon the basis of information not available to him at the time the current Outline of Production for a biological product was filed, object to the methods or procedures being used in the preparation of such biological product and notify the licensee to modify the filed Outline of Production to eliminate such objections. If the licensee does not comply with the notice, the Administrator may, after affording opportunity for a hearing to the licensee, suspend the product license for the biological product involved; in which case, the licensee shall not prepare such product until subsequent notice of withdrawal

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of the suspension is given to the licensee.

(Approved by the Office of Management and Budget under control number 0579-0013)

[39 FR 16869, May 10, 1974, as amended at 48 FR 57473, Dec. 30, 1983; 56 FR 66784, Dec. 26, 1991; 75 FR 20773, Apr. 21, 2010]

§ 114.9 Outline of Production guidelines.

Each Outline of Production shall be prepared in accordance with the applicable directions provided in this section.

(a) *General requirements.* (1) All copies of each Outline of Production or special outline or revised pages of either shall be prepared on heavy paper (8.5"x11") of a type receptive to permanent stamp ink.

(2) The name of the biological product (or component), the establishment license number, and the date prepared shall appear on a front cover page and each page of the Outline of Production or special outline. The name of the licensee (or foreign manufacturer) shall appear on the front cover page.

(3) The pages shall be numbered in the upper center. At least 1½ inch margin shall be left at the top of the first page and a 2 inch margin at the bottom of each page for the Animal and Plant Health Inspection Service stamp.

(4) Amended pages shall be numbered the same as those being superseded. They shall bear the date prepared and refer to the date on the pages being superseded. If one replacement page supersedes more than one page, the new page shall indicate same, but if several replacement pages are added to supersede one page, the page number followed by letters shall be used.

(5) The last page of both copies of either a new or a completely rewritten Outline of Production and each page revised separately shall be signed in the lower left corner by the authorized representative of the licensee (or foreign producer). Stamped or facsimile signatures are not acceptable.

(6) A summary of changes shall appear on an attached page and refer to each page, paragraph, or subparagraph being changed.

(7) Transmittal forms shall be used for the original and subsequent revisions. Transmittal forms are available

on the Internet at (http://www.cphis.usda.gov/animal_health/vet_biologics/vb_forms.shtml).

(b) *Special outline.* An outline describing the preparation of a component of a biological product or an operation performed in the preparation of a biological product may be required if such special outline could be referred to in Outlines of Production to eliminate repetition. Each special outline shall be identified by number and shall not be used until accepted and filed by Animal and Plant Health Inspection Service.

(c) Outline of Production for antiserum, antitoxin, and normal serum shall be written according to the following:

OUTLINE GUIDE FOR PRODUCTION OF ANTISERUM AND ANTITOXIN AND NORMAL SERUM

License No.	Name of Product	Date
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I. *Serum animals.* A. Species, conditions, age, and general health.

B. Examination, preparation, care, quarantine, tests, and treatment of animals before injections are started.

C. Holding, handling, exercising, and monitoring the condition of animals after injections are started.

II. *Antigens.* A. Composition and character of the antigen.

1. Micro-organisms.

2. Source and date of accession of each micro-organism.

3. Strains.

4. Proportions of each micro-organism and strain.

B. Identification methods used for each micro-organism and frequency with which these methods are applied.

C. Virulence and purity of cultures or antigen and the determination and maintenance thereof. Range of subcultures or passages to be used in production.

D. Attenuation, if any, before use for production purposes.

E. Character, size, and shape of containers used for growing micro-organisms.

F. Media used for stock, seed, and antigen cultures (composition and reaction of). May refer to a special outline by number.

G. Preparation of the antigen or toxin and toxoid. Complete and full description of each step and its manner of accomplishment and number these steps in sequence. Include all tests for each antigen, and the specifications for character, identity, virulence, concentration, and standardization.

III. *Immunization of animals.* A. Outline fully with special attention given to the following:

1. Character and dose of the antigen.

2. Method and frequency of injections.
3. Time required for immunization or hyperimmunization.

4. Preliminary bleedings and tests, if any, to ascertain quality of serum.

5. All other similar matters, including treatments between bleedings.

B. Period of time elapsing between last injection and first bleeding; and between bleedings.

C. Technique of bleeding operations; volume of blood collected at each bleeding; and period of rest.

IV. *Preparation of the biological product.* A. Describe fully and show each step of preparation from the first bleeding to the completion of the preserved product in bulk containers prior to filling of final containers.

B. Composition of the preservative and proportions used. Indicate at which step of production, and the method used in adding the preservative.

C. Agglutination and complement-fixation titers and the methods of their determinations.

D. Disposition of unsatisfactory biological products and infective materials not used in production.

E. Assembly of units to make a serial; volume of the average serial; and the volume of the maximum serial.

V. *Testing.* Indicate the stages in the preparation of the biological product at which samples are collected. Refer to all applicable Standard Requirements. Outline all additional tests in detail and state minimum requirements for each satisfactory test.

A. Purity.

B. Safety.

C. Potency.

D. Other tests.

VI. *Post preparatory steps.*

A. Form and size of final containers in which the product is to be distributed.

B. Methods and techniques of filling final containers. Volume of fill for each size final container.

C. Collection, storage, and submission of representative samples. Indicate at which steps in the production these samples are taken.

D. Expiration date based on the earliest date of harvest and the date of the last satisfactory potency test.

E. Use, dosage, and route of administration for each animal species for which it is recommended.

F. Include any additional pertinent information.

(d) Outline of Production for *vaccines, bacterins, antigens, and toxoids* shall be written according to the following:

OUTLINE GUIDE FOR VACCINES, BACTERINS,
ANTIGENS, AND TOXOIDS

License No. Name of Product Date

I. *Composition, etc., of the product.* A. Micro-organisms used. Give the isolation and passage history.

B. Source and date of accession of each micro-organism.

C. Strains.

D. Proportions of each strain.

II. *Cultures.* A. Brief description of methods of identifying each micro-organism and the frequency with which these methods are applied.

B. Virulence and purity of cultures and the determination and maintenance thereof. Range of subcultures or passages to be used in production.

C. Composition and reaction of media used for seed and production cultures. Include the source of eggs, tissue, or cell cultures, and the tests to determine that eggs, tissues, and cells are free of contamination.

D. Character, size, and shape of containers used for growing cultures.

E. Storage conditions of seed cultures.

F. Methods of preparing suspensions for seeding or inoculation.

G. Technique of inoculating (1) seed media; (2) production media. Titer or concentration of inoculum, and the volume of medium for each size and type of culture container.

H. Period of time and conditions for incubation and degree of temperature used for each micro-organism or group of micro-organisms.

I. Character and amount of growth; observation as to contamination of growth.

J. Method of attenuation, if any, before used for production purposes.

III. *Harvest.* A. Handling and preparation of cultures and media (including eggs) before removal of micro-organisms or tissues for production purposes.

B. Minimum and maximum period of time elapsing from time of inoculation until harvest.

C. Technique of harvesting micro-organisms or tissues (specify) for production purposes.

D. Specifications for acceptable harvest material.

E. Handling of discarded material not used in production.

F. Include any additional pertinent information.

IV. *Preparation of the product.* Describe fully and show each step of preparation from harvest of antigen containing tissues or production cultures to the completion of the finished product in final containers. In describing the preparation of the product, emphasize the following:

A. Method of inactivation, attenuation, or detoxification.

B. Composition of preservative, adjuvant or stabilizer, and proportions used stated in such a manner that the concentration can be calculated; stage and method of addition.

C. Method and degree of concentration.

D. If product is standardized to give concentration of antigen, show procedures and calculations.

E. 1. Assembly of units to make a serial (illustrate by example).

2. Volume of average serial.

3. Volume of maximum serial.

4. Any other pertinent information.

F. Volume of fill for each size vial. Type of vial if unusual.

G. Method and technique of filling and sealing of final containers.

H. Desiccation, including moisture control. Give maximum percent moisture.

I. Amount of antigenic material per dose or doses in final container.

V. *Testing*. Indicate the stages in the preparation of the biological product at which the samples are collected. Refer to all applicable Standard Requirements. Outline all additional tests in detail and state the minimum requirement for each satisfactory test.

A. Purity.

B. Safety.

C. Potency.

D. Moisture, if desiccated.

E. Any other tests.

VI. *Post preparatory steps*. A. Form and size of final containers in which the product is to be distributed.

B. Collection, storage, and submission of representative samples. Indicate at which steps in the production these samples are taken.

C. Expiration date based on the earliest date of harvest and the date of the last satisfactory potency test. If applicable, give the date of lyophilization.

D. Use, dosage, and route of administration for each animal species for which the biological product is recommended.

(e) Outlines of Production for allergenic extracts shall be written according to the following:

OUTLINE GUIDE FOR ALLERGENIC EXTRACTS

License No. Name of Product Date

I. *Composition of the product*. A. Source and type of raw material.

B. Weight/volume concentration.

II. *Preparation of the product*. A. Describe fully and show each step of preparation to the completion of the finished product in true containers. In describing the preparation of the product, emphasize the following:

1. Method of extraction.

2. Composition of preservative, adjuvant or stabilizer, and proportions used; stage and method of addition.

3. Method and degree of concentration.

4. Standardization of the product.

5. (a) Assembly of units to make a serial.

(b) Volume of average serial.

(c) Maximum serial.

6. Volume of fill for each size vial.

7. Method and technique of filling and sealing of final containers.

8. Amount material per dose or doses in final container.

III. *Testing*. Indicate the stages in the preparation of the biological product at which the samples are collected. Refer to all applicable Standard Requirements. Outline all additional tests in detail and state the minimum requirement for each satisfactory test.

A. Purity.

B. Safety.

C. Potency.

D. Any other tests.

E. Include any additional pertinent information.

IV. *Post preparatory steps*. A. Form and size of final containers in which the product is to be distributed.

B. Collection, storage, and submission of representative samples. Indicate at which steps in the production these samples are taken.

C. Expiration date based on the earliest date of harvest and the date of the last satisfactory potency test.

D. Use, dosage, and route of administration for each animal species for which the biological product is recommended.

(f) Outlines of Production for diagnostic test kits based on antigen-antibody reactions, and other diagnostics whose production methods are amenable to description as described herein shall be written according to the following requirements:

OUTLINE GUIDE FOR DIAGNOSTIC TEST KITS

License No. Name of product Date

Introduction

Provide a brief description of the kit as follows:

1. Principle of the test (ELISA, latex agglutination, etc.).

2. Antigen or antibody detection test.

3. Sample(s) used for testing (serum, whole blood, tears, etc.).

4. List reagents, references, and equipment included.

5. Identify materials obtained under split manufacturing agreements.

6. General description of test interpretations and their limitations, including followup tests.

I. Antibody Components

A. Production of polyclonal antibody components.

1. If purchased, list suppliers, criteria for acceptability, and describe all tests performed after receipt to determine that specifications have been met.

2. If produced in-house, describe the species, age, weight, conditions, and general health of all animals used in antiserum production.

a. Preinjection considerations:

Describe the examination, preparation, care, quarantine procedures, and treatments administered before immunization(s). Describe all tests used to determine suitability for use. Describe the preparation of any standard negative serum(s) collected prior to immunization.

b. Immunization of animals.

i. Describe the character and dose of the antigen; if adjuvant is used provide details on its preparation. If commercial product is used include its true name as shown on the label, the manufacturer, serial number, and expiration date.

ii. Describe the method and schedule for immunizations.

iii. Describe the method for harvesting and evaluating the immunization product, including tests for acceptability.

iv. Provide number and intervals between harvests, volume obtained, and any other pertinent information.

B. Production of Monoclonal Antibody Components.

1. Hybridoma components:

a. If hybridoma components are purchased, list suppliers and criteria for acceptability; if tests are performed after receipt, describe fully.

b. If hybridomas are prepared inhouse, identify the antigen(s) used, describe the immunization scheme, and the species of animal used.

c. Identify the tissue of origin, and the procedures for harvesting, isolating, and identifying the immune cells.

d. Describe the source, identity, and the product secreted (light or heavy chain) by the parent Myeloma Cell Line.

e. Summarize cloning and recloning procedures, including clone characterization and propagation, if appropriate.

f. If appropriate, describe procedures for establishing and maintaining seed lots.

g. Describe any other pertinent tests or procedures performed on the hybridoma cell line.

2. Antibody production:

a. Describe the production method. If produced in cell culture, animal serum additives must conform to 9 CFR 113.53. If produced in animals, describe fully including husbandry practices and passage procedures.

b. Provide the criteria for acceptable monoclonal antibody, including tests for purity.

c. Describe all tests or other methods used to ensure uniformity between production lots of monoclonal antibody. Include all reaction conditions, equipment used, and reactivity of the component.

d. Describe all characterization procedures and include the expected reactivity of all reference monoclonal antibodies.

II. Antigen Preparation

A. Identify the microorganism(s) or antigen being used. If previously approved Master Seed virus, bacteria, or antigen derived therefrom is used, provide pertinent information on the testing performed, and details of dates of United States Department of Agriculture confirmatory tests and approval, as appropriate.

B. Describe all propagation steps, including identification of cell cultures, media ingredients, cell culture conditions, and harvest methods. For antigen produced in eggs, give the egg source, age, and route of inoculation. If cell lines are being used, give dates of testing and approval as specified in 9 CFR 113.52.

C. Describe procedures used for extracting and characterizing the antigen.

D. Describe the method used to standardize the antigen.

E. If the antigen is purchased, identify the supplier and describe the criteria for acceptable material, including all tests performed by the producer and/or the recipient to determine acceptability.

III. Preparation of Standard Reagents

A. Describe the positive and negative controls included in the kit. If purchased, list suppliers and criteria for acceptance.

B. Describe the preparation and standardization of the conjugate(s). If purchased, list suppliers and criteria for acceptance.

C. Describe the preparation and standardization of the substrate(s). If purchased, list suppliers and criteria for acceptance.

D. Identify buffers, diluents, and other reagents included in the kit. The preparation of these components may be described in this section or in filed Special Outlines.

IV. Preparation of the Product

Fully describe methods used to standardize antigens, reference standards, positive control serum, negative control serum, and standard reagents from production/purchase to completion of finished product in final containers, including the following:

1. Composition and quantity of preservative in each.

2. Method of filling, plating, or attaching the antigen or antibody component to a solid phase.

3. Minimum and maximum acceptable fill volumes for each final container of reagent included in the kit.

4. The disposition of unsatisfactory material.

V. *Testing*

Refer to all applicable standard requirements.

A. *Purity.*

Describe all tests of the kit for purity or specify the exemption as provided in 9 CFR 113.4.

B. *Safety.*

In vitro products are exempt from safety tests.

C. *Potency.*

Provide details of tests used to determine the relative reactivity of the kit including minimum requirements for a satisfactory test. Reference standards and control serum used for this purpose should be identified by unique codes or lot numbers.

VI. *Postpreparatory Steps*

A. Describe the form and size of final containers of each reagent/component included in the kit.

B. Describe the collection, storage, and submission of representative samples. Refer to 9 CFR 113.3(b)(7).

C. Specify the expiration date. Refer to 9 CFR 114.13.

D. Provide details of recommendations for use, including all limitations, qualifications, and interpretation of results.

E. Submit confidentiality statement identifying specific parts of the outline containing information, the release of which would cause harm to the submitter.

(Approved by the Office of Management and Budget under control number 0579-0013)

[39 FR 16869, May 10, 1974, as amended at 48 FR 57473, Dec. 30, 1983; 56 FR 20124, May 2, 1991; 56 FR 66784, Dec. 26, 1991; 75 FR 20773, Apr. 21, 2010]

§ 114.10 **Antibiotics as preservatives.**

Antibiotics are authorized for use as preservatives for biological products if used within the limitations as to kinds and amounts prescribed in this section.

(a) When an antibiotic or combination of antibiotics, with or without a fungistat is to be used in the preparation of a biological product, the kind(s) and amount(s) of each shall be specified in the outline for such product in such a way that the concentration in the final product may be calculated. Except as may be approved by the Administrator, only those individual antibiotics or combinations of antibiotics listed in paragraphs (b) and (c) of this section shall be used.

(b) *Permitted individual antibiotics:*

(1) The antibiotic level of a specified individual antibiotic in one ml. of a bi-

ological product, when prepared as recommended for use, shall not exceed the amounts listed in this paragraph: *Provided*, That in the case a desiccated biological product is to be used with an indefinite quantity of water or other menstruum, the determination shall be based on 30 ml. per 1,000 dose vial or equivalent.

(2) Except as prescribed in paragraph (c) of this section, only one antibiotic shall be used as a preservative in a biological product. The kind and maximum amount per ml. of such antibiotic shall be restricted to:

Amphotericin B	2.5 mcg.
Nystatin (Mycostatin)	30.0 units
Tetracyclines	30.0 mcg.
Penicillin	30.0 units
Streptomycin	30.0 mcg.
Polymyxin B	30.0 mcg.
Neomycin	30.0 mcg.
Gentamicin	30.0 mcg.

(c) *Permitted combinations:*

(1) Penicillin and streptomycin.

(2) Either amphotericin B or nystatin, but not both, may be used with one of the other antibiotics listed in paragraph (b) of this section, or with a combination of penicillin and streptomycin, or with a combination of polymyxin B and neomycin.

(3) The maximum amount of each antibiotic in a combination shall be the amount prescribed for such antibiotic in paragraph (b) of this section.

(d) Antibiotics used in virus seed stock purification are not restricted as to kind or amounts provided carryover into the final product is controlled and specified in outlines of production.

[39 FR 16869, May 10, 1974, as amended at 56 FR 66784, Dec. 26, 1991]

§ 114.11 **Storage and handling.**

Biological products at licensed establishments shall be protected at all times against improper storage and handling. Completed product shall be kept under refrigeration at 35 ° to 45 °F. (2 ° to 7 °C.) unless the inherent nature of the product makes storage at a different temperature advisable, in which case, the proper storage temperature shall be specified in the filed Outline of Production. All biological products to be shipped or delivered shall be securely packed.

§ 114.12 Expiration date required.

Each serial or subserial of biological product prepared in a licensed establishment shall be given an expiration date determined in accordance with the requirements provided in § 114.13 or § 114.14. A licensed biological product shall be considered worthless under the Virus-Serum-Toxin Act subsequent to the expiration date appearing on the label.

[41 FR 44687, Oct. 12, 1976]

§ 114.13 Expiration date determination.

Unless otherwise provided for in a Standard Requirement of filed Outline of Production, the expiration date for each product shall be computed from the date of the initiation of the potency test. Prior to licensure, stability of each fraction shall be determined by methods acceptable to Animal and Plant Health Inspection Service. Expiration dates based on this stability data shall be confirmed as follows:

(a) *Products consisting of viable organisms.* Each serial shall be tested for potency at release and at the approximate expiration date until a statistically valid stability record has been established.

(b) *Nonviable biological products.* Each serial presented in support of licensure shall be tested for potency at release and at or after the dating requested.

(c) Subsequent changes in the dating period for a product may be granted, based on statistically valid data submitted to support a revision of the Outline of Production.

[50 FR 24903, June 14, 1985, as amended at 56 FR 66784, Dec. 26, 1991]

§ 114.14 Extension of expiration date for a serial or subserial.

(a) Unless otherwise provided for in a filed Outline of Production for the product, the expiration date shall not be extended:

(1) If all fractions of the product are not evaluated for potency by tests designated in the filed Outline of Production for such product in accordance with § 113.4(b) of this subchapter.

(2) For any serial or portion of any serial which has left licensed premises: *Provided*, That product which has been

shipped from one licensed premises to another licensed premises shall be exempt from this requirement.

(3) For a serial or portion of a serial if the expiration date has been extended previously, unless otherwise authorized in accordance with § 114.1.

(b) An extension of the expiration date may be granted by Animal and Plant Health Inspection Service if a request from the licensee is substantiated by valid test data which demonstrate the potency of the product meets or exceeds the requirements for release. The new expiration date shall be calculated from the date the latest satisfactory potency test was initiated. The extension of the expiration date shall not exceed the maximum dating allowed in the filed Outline of Production.

(1) Serials are approved for redating under the condition that Animal and Plant Health Inspection Service may require the firm to retest the redated serial for potency during the extended dating period and if found unsatisfactory require it be removed from the market by the licensee.

(2) [Reserved]

[50 FR 24903, June 14, 1985, as amended at 56 FR 66784, Dec. 26, 1991]

§ 114.15 Disposal of unsatisfactory products and byproducts.

All biological products found to be unsatisfactory for marketing, all biological products which have become worthless subsequent to the expiration date, all refuse, other materials deemed unsatisfactory for production purposes, all carcasses (part or whole) of production or test animals, and any undesirable byproducts of manufacture shall be disposed of as may be required by the Administrator.

[41 FR 44687, Oct. 12, 1976, as amended at 56 FR 66784, Dec. 26, 1991]

§ 114.16 Producing subsidiaries.

A serial or subserial of a biological product may be produced jointly by a licensee and one or more subsidiaries, or by two or more subsidiaries. The exact amount of each serial or subserial credited to each participating producer shall be determined at the time of labeling and packaging and

§ 114.17

shall be noted in the records for such serial or subserial.

[40 FR 46093, Oct. 6, 1975]

§ 114.17 Rebottling of biological products.

The Administrator may authorize the rebottling of a completed product in liquid form subject to the conditions prescribed in this section.

(a) All or part of a serial which has not left the licensed establishment may be aseptically returned to the mixing tank, thoroughly mixed, and rebottled in new final containers.

(b) The rebottled product shall be adequately identified by serial number or subserial number, as the case may be.

(c) Required purity tests for final container samples of the product shall be conducted on new samples selected from the rebottled product (serial or subserials). Rebottled product found to be unsatisfactory by such tests shall not be released.

(d) New test samples from each serial or subserial and copies of test reports of all tests conducted on the rebottled product shall be submitted to Animal and Plant Health Inspection Service.

(e) The licensee shall not release the rebottled product unless notified by Animal and Plant Health Inspection Service that such product is eligible for release. Production records shall show the results of all tests conducted and shall accurately reflect the actions taken.

[39 FR 16869, May 10, 1974, as amended at 56 FR 66784, Dec. 26, 1991]

§ 114.18 Reprocessing of biological products.

The Administrator may authorize a licensee to reprocess a serial of completed product subject to the conditions prescribed in this section.

(a) Reprocessing shall not include any method or procedure which would be deleterious to the product.

(b) All appropriate tests for purity, safety, potency, and efficacy for the product shall be conducted on the reprocessed product. A serial found unsatisfactory by a required test shall not be released.

(c) The reprocessed serial shall be identified by a new serial number and

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the records for the serial shall accurately reflect the action taken.

(d) Test samples of the reprocessed serial and test reports for all tests conducted shall be submitted to Animal and Plant Health Inspection Service. The licensee shall not release the serial until notified by Animal and Plant Health Inspection Service that the serial is eligible for release.

[50 FR 24904, June 15, 1985, as amended at 56 FR 66784, Dec. 26, 1991]

PART 115—INSPECTIONS

Sec.

115.1 Inspections of establishments.

115.2 Inspections of biological products.

AUTHORITY: 21 U.S.C. 151-159; 7 CFR 2.22, 2.80, and 371.4.

§ 115.1 Inspections of establishments.

(a) Any inspector shall be permitted to enter any establishment where any biological product is prepared, at any hour during the day or night, and shall be permitted to inspect, without previous notification, the entire premises of the establishment, including all buildings, compartments, and other places, all biological products, and organisms and vectors in the establishment, and all materials and equipment, such as chemicals, instruments, apparatus, and the like, and the methods used in the manufacture of, and all records maintained relative to, biological products produced at such establishment.

(b) Each inspector will have in his or her possession a numbered USDA badge or identification card. Either shall be sufficient identification to entitle him/her to admittance at all regular entrances and to all parts of such establishment and premises and to any place at any time for the purpose of making an inspection pursuant to paragraph (a) of this section.

[52 FR 30134, Aug. 13, 1987]

§ 115.2 Inspections of biological products.

(a) Any biological product, the container of which bears a United States veterinary license number or a United States veterinary permit number or

London, 18 September 2007
Doc. Ref. EMEA/CVMP/VICH/1052/2004

VICH Topic GL41

at Step 7

**GUIDELINE ON TARGET ANIMAL SAFETY: EXAMINATION OF LIVE VETERINARY
VACCINES IN TARGET ANIMALS FOR ABSENCE OF REVERSION TO VIRULENCE**

ADOPTION BY CVMP FOR RELEASE FOR CONSULTATION	10 November 2004
TRANSMISSION TO INTERESTED PARTIES	15 November 2004
END OF CONSULTATION	10 May 2005
SIGN-OFF AT STEP 6 BY STEERING COMMITTEE	August 2007
ADOPTION BY CVMP	13 September 2007
DATE FOR COMING INTO EFFECT	1 July 2008

VICH GL 41 (TARGET ANIMAL SAFETY) – REVERSION TO VIRULENCE

July 2007

For implementation at Step 7

TARGET ANIMAL SAFETY: EXAMINATION OF LIVE VETERINARY VACCINES IN TARGET ANIMALS FOR ABSENCE OF REVERSION TO VIRULENCE

Recommended for Adoption
at Step 7 of the VICH Process
in July 2007 by the VICH SC
for implementation in July 2008

This Guideline has been developed by the appropriate VICH Expert Working Group and is subject to consultation by the parties, in accordance with the VICH Process. At Step 7 of the Process the final draft will be recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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1. INTRODUCTION

The absence of reversion to or increase in virulence test is generally an essential requirement for the registration or licensure of live vaccines in the EU, Japan and the USA. International harmonization of this test will minimize the need to perform separate studies for regulatory authorities of different countries. Appropriate international standard methods will reduce research and development costs by avoiding, whenever possible, duplication of tests. Animal welfare will benefit because fewer animals will be needed by eliminating repetition of similar tests in each region.

This guideline has been developed under the principle of VICH and will provide a unified standard for government regulatory bodies to facilitate the mutual acceptance of reversion to virulence data by the relevant authorities. The use of this VICH guideline to support registration of a product for local distribution only is strongly encouraged but is up to the discretion of the local regulatory authority. Furthermore, it is not always necessary to follow this guideline when there are scientifically justifiable reasons for using alternative approaches.

1.1. Objective

This guideline establishes agreed criteria and requirements for the conduct of studies that examine the potential for reversion to or increase in virulence of live veterinary vaccines in target animals.

1.2. Scope and General Principle

This guideline is intended to cover live vaccines. Live vaccines ⁽¹⁾ are those that may be capable of replication in the target animal, stimulate a useful immune response, and generally cannot be completely characterized by chemical and physical tests alone. The guideline covers the following species: bovine, ovine, caprine, feline, canine, porcine, equine, poultry (chickens and turkeys). This guideline will not provide information for the design of tests in other species including aquatic animals. For other species, tests should be designed following local guidance. Guidance on laboratory tests to determine adequate attenuation of the vaccine strain is not within the scope of this guideline.

(1) In case of vector vaccines this only covers vector vaccine seeds that replicate in the target species.

2. STUDY DESIGN

This study is carried out using the master seed. If the quantity of the master seed sufficient for testing is not available, the lowest passage seed used for production that is available in sufficient quantity should be examined. Use of another passage option must be justified. Generally, serial passages should be made in target animals through five groups of animals, unless there is justification to make more passages or the organism disappears from the test animals sooner. The time interval between inoculation of the animal and harvest for each passage must be justified based upon the characteristics of the test organism. If recovery is successful, passages should continue through five groups of animals. Appropriate methods, preferably *in vitro* propagation, should be used to confirm the presence and to determine the number of the test organisms at each passage. *In vitro* propagation may not be used to expand the passage inoculum.

Where a reasonable explanation for the sudden loss of the organism exists, e.g. experimental error, the previous passage may be repeated. When the organism is not recovered from any intermediate *in vivo* passage, a reasonable attempt should be made to repeat the test in 10 animals (90% probability of isolating the organism at 20% probability of recovery – see Appendix) using *in vivo* passaged material from the last passage in which the organism was recovered. If the target organism is recovered from one or more animals in the repeat test, the passages should continue using the material recovered in the repeat test as the inoculum for the next passage. The repeat test will be counted as a passage. If the target organism is not recovered, the experiment is considered to be completed with the conclusion that the target organism does not show an increase in or reversion to virulence.

Generally, for each target species, the most sensitive class, age, sex and serological status of animals should be used. In cases where alternative approaches are used, alternatives should be justified. Generally, a minimum of two animals is used for the first four groups and a minimum of eight animals is used for the fifth group.

Housing and husbandry should be adequate for the purpose of the study and conform to local animal welfare regulations. Animals should be appropriately acclimatized to the study conditions. Appropriate prophylactic treatment should be completed before the initiation of the study. Reduction or elimination of suffering during the study is essential. Euthanasia and necropsy of moribund animals is recommended.

The initial administration and subsequent passages shall be carried out using a recommended route of administration or natural route of infection that is the most likely to lead to reversion to or increase in virulence and result in recovery of the organism following replication in the animal. The route used must be justified.

The initial inoculum should contain the maximum release titer expected in the recommended dose or, in the cases where the maximum release titer to be licensed is not specified, then a justifiable multiple of the minimum release titer can be used. Passage inocula should be collected and prepared from the most likely source of spread of the organism, unless there is scientific justification to use another material.

General clinical observations should be made during the study. Animals in the fifth group should be observed for 21 days unless otherwise justified. These observations should include all relevant parameters typical for the disease which could indicate reversion to or increase in virulence. If signs consistent with the target disease are observed, then causality needs to be investigated. No evidence of an increase in virulence, indicative of reversion, should be seen with passage.

If the fifth group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, materials used for the first passage and the final passage should be used in a separate experiment using at least 8 animals per group to directly compare the clinical signs and other relevant parameters. This study should be done by the route of administration that was used for previous passages. An alternative route of administration may be used if scientifically justified.

When attenuation of a test organism is known to be the result of a well characterized specific marker or genetic change, additional tests using suitable molecular biological methods for comparison of the initial seed organism and the organism recovered from the final passage should be performed, thus confirming the genetic stability of the attenuation marker in the vaccine strain.

If available data or assessment indicate a substantial risk that the test organism may revert to or increase in virulence, additional studies may be required to provide further information on the organism.

Except in exceptional and justified cases, if the completed studies show that the test organism does revert to or increase in virulence after passage in the target animal, the test organism will be deemed to be unsuitable for use as a live vaccine.

3. GLOSSARY

Class. Subset of target animal species which is characterized by factors such as reproductive status and/or use (dairy vs. beef, broiler vs. layer)

Master seed. A collection of aliquots of a micro-organism suspension for use in the preparation of the product, obtained from single culture, distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability.

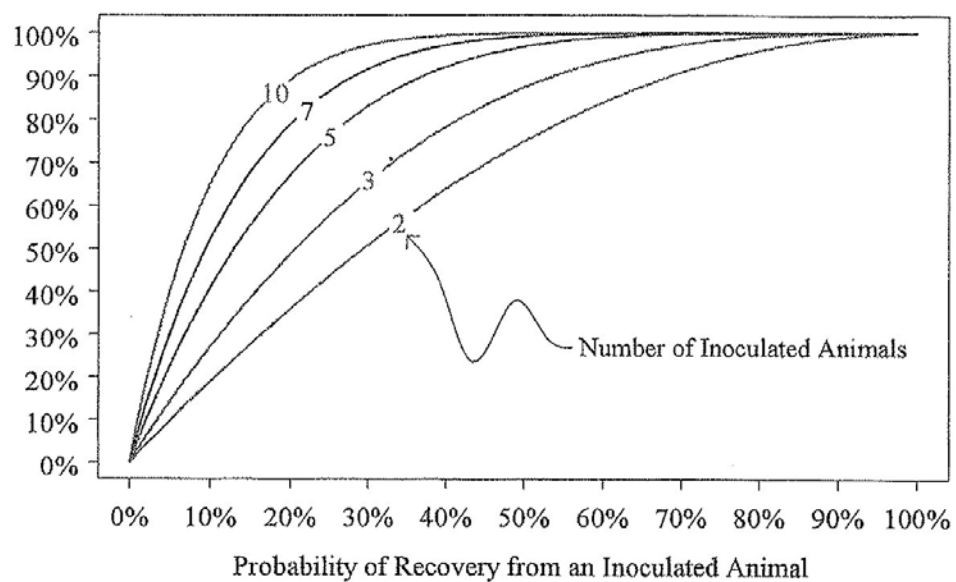
Maximum release titer. The expected highest number of viable organisms allowed per dose in vaccines at the time of release, verified by safety studies. In regions where a maximum release potency is not established, a justifiable multiple of the release antigen content is applied.

Minimum release titer. The expected lowest number of viable organisms required per dose in vaccines at the time of release, verified by efficacy and stability data.

Passage. Transfer of organisms through a group of inoculated animals, either from the beginning seed material or from a previous passage in animals.

APPENDIX

Probability of Recovery from At Least One Animal of a Group of Inoculated Animals



APPENDIX

The table gives the probability that the organism will be recovered from at least one animal of a group of inoculated animals. The probability of recovery from a single animal is in the left margin. The number of inoculated animals is in the top margin. The corresponding entry shows the probability that the organism will be recovered from at least one animal in the group.

Probability of Recovery from an Inoculated Animal	Number of Inoculated Animals				
	2	3	5	7	10
0.975	0.999	>0.999	>0.999	>0.999	>0.999
0.950	0.997	>0.999	>0.999	>0.999	>0.999
0.925	0.994	>0.999	>0.999	>0.999	>0.999
0.900	0.990	0.999	>0.999	>0.999	>0.999
0.875	0.984	0.998	>0.999	>0.999	>0.999
0.850	0.978	0.997	>0.999	>0.999	>0.999
0.825	0.969	0.995	>0.999	>0.999	>0.999
0.800	0.960	0.992	>0.999	>0.999	>0.999
0.775	0.949	0.989	0.999	>0.999	>0.999
0.750	0.938	0.984	0.999	>0.999	>0.999
0.725	0.924	0.979	0.998	>0.999	>0.999
0.700	0.910	0.973	0.998	>0.999	>0.999
0.675	0.894	0.966	0.996	>0.999	>0.999
0.650	0.878	0.957	0.995	0.999	>0.999
0.625	0.859	0.947	0.993	0.999	>0.999
0.600	0.840	0.936	0.990	0.998	>0.999
0.575	0.819	0.923	0.986	0.997	>0.999
0.550	0.798	0.909	0.982	0.996	>0.999
0.525	0.774	0.893	0.976	0.995	0.999
0.500	0.750	0.875	0.969	0.992	0.999
0.475	0.724	0.855	0.960	0.989	0.998
0.450	0.698	0.834	0.950	0.985	0.997
0.425	0.669	0.810	0.937	0.979	0.996
0.400	0.640	0.784	0.922	0.972	0.994
0.375	0.609	0.756	0.905	0.963	0.991
0.350	0.577	0.725	0.884	0.951	0.987
0.325	0.544	0.692	0.860	0.936	0.980
0.300	0.510	0.657	0.832	0.918	0.972
0.275	0.474	0.619	0.800	0.895	0.960
0.250	0.437	0.578	0.763	0.867	0.944
0.225	0.399	0.535	0.720	0.832	0.922
0.200	0.360	0.488	0.672	0.790	0.893
0.175	0.319	0.438	0.618	0.740	0.854
0.150	0.277	0.386	0.556	0.679	0.803
0.125	0.234	0.330	0.487	0.607	0.737
0.100	0.190	0.271	0.410	0.522	0.651
0.075	0.144	0.209	0.323	0.421	0.541
0.050	0.097	0.143	0.226	0.302	0.401
0.025	0.049	0.073	0.119	0.162	0.224

Routes of entry of *Piscirickettsia salmonis* in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Since 1989, *Piscirickettsia salmonis*, the causal agent of piscirickettsiosis, has killed millions of farmed salmonids each year in southern Chile. The portal of entry for the pathogen was investigated by use of selected experimental infections in juvenile rainbow trout (12 g). The methods used were intraperitoneal injection, subcutaneous injection, patch contact on skin, patch contact on gills, intestinal intubation and gastric intubation. Cumulative mortalities at Day 33 post-inoculation were 98, 100, 52, 24, 24, and 2%, respectively. It was shown that intact skin and gills could be penetrated by *P. salmonis*. The high mortality obtained in subcutaneously injected fish indicated that skin injuries could facilitate the invasion of this pathogen. Results suggested that the main entry sites are through the skin and gills and that the oral route may not be the normal method by which *P. salmonis* initiates infection of salmonids.

KEY WORDS: *Piscirickettsia salmonis* · Pathogenesis · Fish disease · Salmonid

INTRODUCTION

Gram-negative, obligated intracellular, fastidious bacteria have been increasingly detected in a wide range of fish species in different geographic locations, and are currently recognised as an important group of fish pathogens (Fryer & Mauel 1997). *Piscirickettsia salmonis* is the etiological agent of a devastating disease of maricultured salmonids in Chile (Fryer et al. 1990, Cvitanich et al. 1991, Garcés et al. 1991) known at present as 'salmon rickettsial septicaemia' (Cvitanich et al. 1991) or 'piscirickettsiosis' (Fryer et al. 1992). Losses are not limited to fish mortality, but also include other costs such as those for antibacterial drugs. Approximately US\$7.5 million was spent on antimicrobial treatment of bacterial diseases of salmonid species in 1997 in Chile, and most of this was used for therapeutics against piscirickettsiosis (J. Cassigoli, Chilean Salmon Growers Association, pers. comm. 1998). In spite of the extensive use of drugs, the control of this disease has not been successful and losses have increased progressively (Almendras & Fuentealba

1997). Protective vaccines are needed to prevent piscirickettsiosis and diminish the use of antimicrobials. Administration of experimental *P. salmonis* bacterins has provided inconsistent results to date (Smith et al. 1997). Improved understanding of the disease pathogenesis of piscirickettsiosis will be critical to our ability to design more rational and efficient therapeutic and/or immunoprophylactic strategies. As a first step to determine the mechanisms used by the pathogen to gain access to the fish host, we assessed the efficiency of different entry sites of *P. salmonis* in juvenile rainbow trout *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Fish. Rainbow trout (ca 12 g) were obtained from a freshwater commercial farm located in an area where piscirickettsiosis has never been reported (Metropolitan Region of Chile). To confirm the absence of *Piscirickettsia salmonis*, kidney smears were tested by an indirect fluorescence antibody test (IFAT) according to the method of Lannan et al. (1991), as modified by Larenas et al. (1996a). A polyclonal *P. salmonis* anti-serum, kindly provided by Mrs C. N. Lannan, from

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Oregon State University (USA) was used. Fish were kept at a research facility that used an inflow of filtered and ultraviolet (UV) disinfected fresh water. All the experimental trouts, infected and sham-inoculated, were held in 100 l fibreglass tanks (25 fish in each tank) that had aeration (ca 8 mg of oxygen l⁻¹) and were supplied with a flow-through fresh water system (1 l min⁻¹). Fish were fed twice a day at a daily rate of 1% body weight on dry commercial pellets. Water temperature was 16.1°C (SD = 0.6°C). Effluent was UV disinfected and treated with 5 ppm sodium hypochlorite.

Bacterium. The SLGO-95 strain of *Piscirickettsia salmonis* (Mauel et al. 1996, Smith et al. 1996b) at the 9th culture passage was used. The organism was cultured using monolayers of the CHSE-214 cell line (ATCC CRL 1681) (Lannan et al. 1984). Cells were grown in Eagle's Minimum Essential Medium with Earle's salts (Automod, Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% foetal calf serum (MEM-10) (Gibco BRL, Grand Island, NY, USA) in the absence of antibiotics and antimycotics. Cell cultures inoculated with *P. salmonis* were incubated at 17°C until the cytopathic effect reached approximately 100%. Infectious supernatants were titrated by end point dilution assay (TCID₅₀ ml⁻¹) in 96-well plates with 6 wells per dilution. Dilution end points were calculated using the method of Reed & Muench (1938).

Infectivity study using different entry routes. Fish were infected with *Piscirickettsia salmonis* using 6 different entry sites to evaluate their eventual efficiency in reproducing the disease by means of the comparison of their cumulative mortality and survivability. Experimental fish were distributed into 13 groups, 50 individuals each, which were allotted into two 100 l fibreglass tanks (25 fish in each tank). Each infected fish group had a corresponding sham-inoculated control group and the remaining one consisted of non-inoculated fish. Fish were sedated with tricaine methyl sulfonate (MS-222, Sigma) before being inoculated. Fish were observed until Day 33 post-inoculation (p.i.). No serial samples were taken to avoid distorting the mortality figures. Each dead fish was examined by necropsy and standard microbiology methods to confirm the cause of the death.

Intraperitoneal injection (IP): Fish were injected IP with 100 µl of a suspension containing 10⁴ TCID₅₀. This group served as a virulence control of inoculum.

Subcutaneous injection (SC): Fish were injected SC in the ventral left side of the body, between the pelvic and the anal fin with 10⁴ TCID₅₀ contained in a volume of 50 µl.

Skin patch (SP): A modification of the method reported by Kanno et al. (1989) was used. Filter papers (Micro Filtration Systems, No. 2, cat. No. 25, Sierra

Court, CA, USA) measuring 49 mm² (square with 7 mm sides) were used. Paper patches were soaked in a rickettsial suspension (10⁶ TCID₅₀ ml⁻¹), and placed on the skin for 1 min on the left side of the fish, directly on the lateral line, in the region located under the dorsal fin. After taking off the patches, fish were held in a bath containing MEM-10 for 30 min, under permanent oxygenation, and afterward returned to their tanks. A titre of 10^{4.2} TCID₅₀ ml⁻¹ was obtained from a bacterial suspension prepared by means of washing a soaked paper patch in 1 ml of MEM-10. This titre could be an approximation of the actual dose to which the fish were exposed with the paper patches.

Gill patch (GP): Gills were infected using the same technique as described in the skin patch infection method. The paper patch was located in the external surface of the first branchial arch of the left side of the fish.

Gastric intubation (GI): An inoculum containing 10⁴ TCID₅₀ in a volume of 200 µl was placed in the stomach, in the transitional region between the cardiac and pyloric region, using a plastic cylindrical flexible probe (external diameter 1.7 mm) with a rounded tip (Infant Feeding Tube, Pennine Healthcare Products, UK) connected to a tuberculin syringe. Fish were fasted for 2 d prior to inoculation.

Intestinal intubation (AI): An inoculum containing 10⁴ TCID₅₀ in a volume of 200 µl was placed inside the descending intestine, through the anal opening, 3 cm from the fish anus by means of the same kind of probe and conditions used in the GI inoculation.

Sequential tissue sampling to study the progression of the bacterium penetration and to evaluate possible cross-infections among entry sites. Another group of 50 fish was inoculated by SP, GP, GI and AI, respectively, to provide serial samples that were examined by IFAT to detect the presence of *Piscirickettsia salmonis*. Examination of the IFAT was performed using 50 microscopic fields at 1000× magnification adopting the method of Elliott & McKibben (1996) developed for quantitative detection of *Renibacterium salmoninarum*. Five fish per group were euthanized by anaesthetic overdose at each sampling time. Samples were taken at 5 min, 15 min, 30 min, 1 h, 2 h, 22 h, 60 h, 7 and 14 d p.i. Skin, gills, stomach and intestine were obtained from the fish of the groups inoculated SP, GP, GI and AI, respectively, at each sampling time. Tissue samples were fixed in 10% buffered formalin (pH = 7.2) embedded in paraffin, and histological cross-sections were processed for IFAT. In addition, kidney smears were taken from all the groups at 60 h and 7 and 14 d p.i. to be used as a marker of systemic infection caused by *P. salmonis*.

In order to determine if cross-infections with *Piscirickettsia salmonis* of other entry sites occurred after

SP and/or GP exposure methods, tissue smears were taken at 5 and 30 min p.i. and examined by IFAT. In the fish inoculated by SP, smears from gill surface and mucosa of stomach and rectum were obtained. In the fish exposed by GP, smears were taken from skin that surrounds the operculum and between the dorsal and pelvic fins, and from the mucosa of stomach and rectum.

RESULTS

Infectivity study

No mortalities or clinical signs of disease were observed in any negative control fish. At Day 33 p.i., cumulative mortalities in experimentally infected fish reached 98% (IP), 100% (SC), 52% (SP), 24% (GP), 2% (GI) and 24% (AI) (Figs. 1 & 2). All diseased fish showed clinical signs and gross pathological features consistent with those described in fish with piscirickettsiosis (Fryer et al. 1990, Cvitanich et al. 1991). Kidney smears from each dead fish was examined by IFAT, and *Piscirickettsia salmonis* was detected in all of them.

All of the fish exposed by SP presented, in addition, skin lesions at the site of inoculation (ca 1 cm²). Gross pathology in some of the fish infected by SP started with haemorrhagic spots on the skin but most cases exhibited a slight raised area as the earliest detectable change, followed by a whitish decoloration with scale loss and induration. Lesions then progressed to ulceration and darkening (Fig. 3). The underlying subcutaneous tissue and skeletal muscle were also haemorrhagic (Fig. 4). At the histological level, these lesions were characterised as ulcerative, affecting the epidermis, stratum spongiosum, stratum compactum and muscular tissue (Fig. 5). Scale loss, leucocyte infiltration

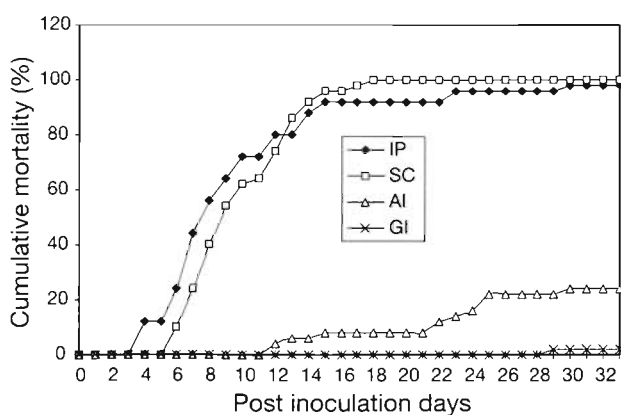


Fig. 1. *Oncorhynchus mykiss*. Cumulative mortality (%) in rainbow trout infected with a dose of 10^4 TCID₅₀ of *Piscirickettsia salmonis* by intraperitoneal injection (IP), subcutaneous (SC), intestinal intubation (AI) and gastric intubation (GI)

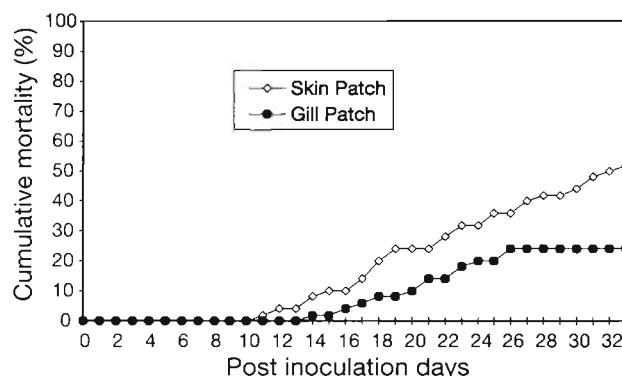


Fig. 2. *Oncorhynchus mykiss*. Cumulative mortality in rainbow trout infected with *Piscirickettsia salmonis* by contact patch in skin and gills

and proliferation of connective tissue was also observed in some areas that presented a nodular appearance.

Statistical comparison of survival probability was done among groups inoculated with the same bacterial doses (i.e. IP, SC, GI and AI, all inoculated with a dose of 10^4 TCID₅₀, and SP and GP, which were exposed with soaked paper patches). Survival data were obtained with the non-parametric method of Kaplan & Meier and compared with the Wilcoxon test (Lee 1992). Survival of fish inoculated IP and/or SC was significantly lower than the ones exposed by AI and/or GI methods ($p < 0.05$). No differences were found between IP and SC injection ($p > 0.05$). In contrast, survival probability of GI was higher than AI ($p < 0.05$). Similarly, analysis showed a higher survivability of fish infected by GP compared with the ones exposed by SP inoculation ($p < 0.05$).

Sequential sampling

Results of the detection of *Piscirickettsia salmonis* by IFAT in cross-sections of serial samples of skin, gills, stomach and intestine from fish inoculated by SP, GP, GI and AI, respectively, are shown in Table 1. Kidney smears from fish inoculated by SC, SP, GP and AI were positive at the 3 sampling times (60 h and 7 and 14 d). No positive infection was found in kidney smears from fish inoculated by GI.

The finding of cross-contamination between entry sites was detected in only 1 case. Small amounts of *Piscirickettsia salmonis* were seen (2 organisms) from the smears of the skin close to the gills at 30 min after the GP inoculation. All the other smears were negative.

DISCUSSION

Rickettsial organisms have diverse morphology, behaviour and pathogenesis. Among their most com-



Fig. 3. *Oncorhynchus mykiss*. Lateral view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact on the skin (scale bar = 0.4 cm). Ulcer at the exposure site (arrow)

mon characteristics are Gram-negative cell walls, an obligatory existence within host cells and a transmission mechanism usually associated with arthropod vectors (Weiss 1982). Most rickettsial agents of terrestrial vertebrates penetrate their final hosts through the skin or mucous membranes via a puncture wound made by arthropod vectors (Campbell 1994). One exception is Q fever, where the causative organism (*Coxiella burnetii*) is quite resistant to the environment. It appears to develop a sporogenic phase that protects it from drying when it is outside a host or vector, and it can be directly transmitted by aerosol (Weiss & Moulder 1984).

With respect to the aquatic Gram-negative intracellular bacteria, with the exception of *Piscirickettsia salmonis*, the source, reservoir and means of transmission of these pathogens are unknown. In the case of *P. salmonis*, some research has been designed to demonstrate the role of horizontal (Cvitanich et al. 1991, Garcés et al. 1991, Pérez et al. 1995, Almendras et al. 1997, Salinas et al. 1997, Smith et al. 1997) or vertical transmission (Larenas et al. 1996b) and to detect potential vectors or reservoirs (Garcés et al. 1994). Cvitanich et al. (1991), Almendras et al. (1997) and Salinas et al. (1997) reported horizontal transmission in different cohabitation experiments. Further evidence of horizontal trans-

mission in the absence of vectors was provided by detecting the bacterium in internal organs after the immersion of fish in *P. salmonis* suspensions. Nevertheless, the disease was not reproduced following these immersion exposures (Pérez et al. 1995, Smith et al. 1997). In a number of experiments, *P. salmonis* has been passed by injection (Cvitanich et al. 1991, Garcés et al. 1991, Smith et al. 1996a), but the normal mode of transmission has not been conclusively demonstrated (Fryer & Mael 1997).

The infectivity experiments reported here indicate that *Piscirickettsia salmonis* can enter its host through a variety of tissues, with the skin being a highly efficient route of entry for this pathogen. This is supported by the high cumulative mortality obtained (52%) and the consistent lesions seen

at the infection site after inoculation by SP. It is apparent that *P. salmonis* is efficient in attaching and invading through the skin as the exposure time was short (1 min), the contact area small (49 mm²) in respect to the total skin surface, and these integumentary tissues were intact at least at the macroscopic level. To determine if skin alone, without the lateral line, allows entry of *P. salmonis*, a subsequent SP inoculation was performed (not explained in 'Materials and methods'—this experiment was done only with 10 exposed and 10 control fish) but the

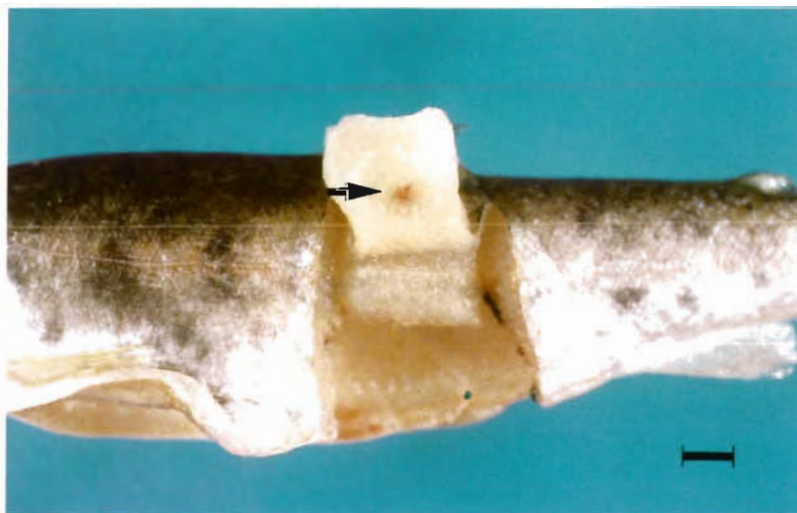


Fig. 4. *Oncorhynchus mykiss*. Lateral view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact on the skin (scale bar = 0.7 cm). Haemorrhage in the skeletal muscle underlying the exposure site (arrow)

Table 1. Detection of *Piscirickettsia salmonis* by IFAT (performed using 50 microscopic fields at 1000× magnification) in histological sections of serial samples of skin, gill, stomach and intestine of rainbow trout following different routes of experimental infection. Values in brackets show the number of positive fish. Five fish were examined, for each route of infection, at a given sampling time.

The IFAT interpretation was: + = 0 to 10 bacteria, ++ = 11 to 50 bacteria, +++ > 50 bacteria, – = negative

Organ	Sampling time								
	5 min	15 min	30 min	1 h	2 h	22 h	60 h	7 d	14 d
Skin (SP)^a									
Epithelium surface	+(2)	+(1)	+(2)	–	+(1)	–	–	–	–
Epidermis	++(1)	+(1)	–	+(1)	+(2)	–	+(1)	–	+++ (1)
Dermis	–	–	+(4)	+(5)	+(2)	+(3)	–	+(2)	+(2), +++ (1)
Skeletal muscle	–	–	–	–	–	–	–	+(1)	+(1), +++ (1)
Gill (GP)^b									
Epithelium surface	+(2), ++ (1)	+(3)	+(4)	+(4)	–	+(2)	–	–	–
Epithelium	+(4)	+(3)	+(1)	+(3)	+(1)	+(2)	+(2)	+(2)	+(2)
Capillar vessels	–	–	–	–	–	–	+(1)	+(2)	+(2)
Stomach (GI)^c									
Lumen	+(2)	+(1)	+(1)	+(1)	–	–	–	–	–
Gastric epithelium	–	–	–	–	+(1)	–	–	+(2)	–
Intestine (AI)^d									
Lumen	+(1)	+(3)	+(2)	–	–	–	–	–	–
Gut epithelium	–	–	+(2)	+(2)	+(1)	–	+(2)	–	–

^aExperimental infection with *P. salmonis* by patch contact on skin

^bExperimental infection with *P. salmonis* by patch contact on gill

^cExperimental infection with *P. salmonis* by gastric intubation

^dExperimental infection with *P. salmonis* by intestinal intubation through the anal opening

exposure site was immediately anterior to the dorsal fin. A similar mortality pattern and pathology were observed (Fig. 6). Therefore, although it is not possible to rule out a role of the lateral line in this matter, the skin by itself is an entrance tissue. Skin lesions

seen were similar to those described for fish infected with *P. salmonis* in farming conditions (Branson & Nieto-Díaz Muñoz 1991) but were not found when the pathogen is injected intraperitoneally (Garcés et al. 1991, Smith et al. 1996a).

Consistent with the gross pathology and the histological findings described in Fig. 5, skin serial samples examined by IFAT showed a progressive penetration by the bacterium to deeper tissues with time (Table 1). The bacterium was even found in muscular tissues at Days 7 and 14 p.i. Nevertheless the number of bacteria detected, in general, was very small, which was not congruous with the magnitude of the lesions observed.

The ability to penetrate the skin is remarkable, because as Evelyn (1996) hypothesised, in the absence of injury, or without the assistance of another parasite such as a leech or louse, invasion via the skin is much more difficult to accomplish because, structurally, the skin presents a far more formidable barrier to penetration than does the gill or intestine. The only previous evidence for bacterial invasion of fish

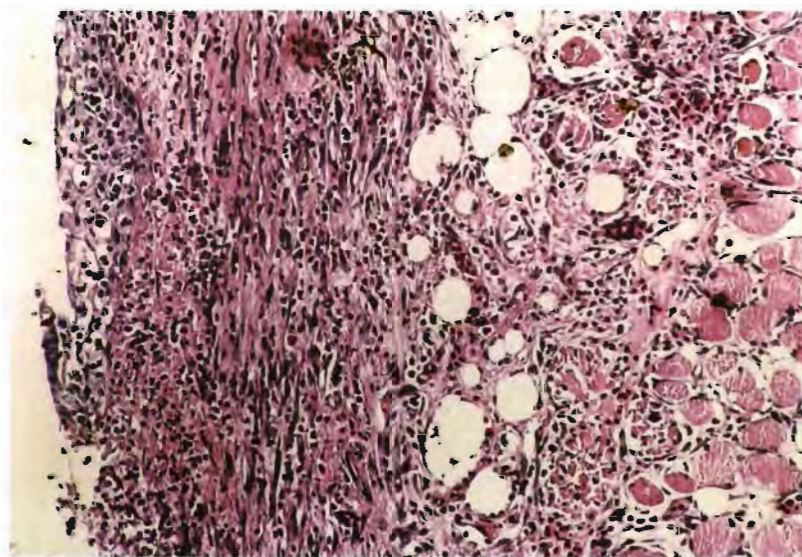


Fig. 5. *Oncorhynchus mykiss*. Skin of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact. Necrosis and infiltration of inflammatory cells in epidermis, dermis and skeletal muscle underlying the exposure site. H&E. 400×



Fig. 6. *Oncorhynchus mykiss*. Dorsal view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact placed on the skin anteriorly to the dorsal fin (scale bar = 0.7 cm). Ulcer and darkening on the skin of the exposure site (arrow)

via intact skin was obtained for *Vibrio anguillarum* in experimental infections of ayu *Plecoglossus altivelis* using the paper patch method (Kanno et al. 1989) that was adopted in this work. Results reported by Effendi & Austin (1995) suggests that the skin may be one of the entry sites of *Aeromonas salmonicida*, but a swabbing method of inoculation was used which could have facilitated bacterial penetration.

The pattern of cumulative mortality among fish injected SC was the same as that among fish inoculated by IP injection, reaching 100 and 98%, respectively (Fig. 1). The expected delay in the onset of the mortalities in fish infected SC relative to the IP method was not observed, and the survivability of the 2 groups was also equal ($p > 0.05$). Although, as discussed previously, *Piscirickettsia salmonis* can enter intact skin, results of the SC injection suggested that skin injuries could greatly increase the likelihood of penetration of the pathogen into the host. Mechanical skin damage, frequently observed in cultured salmon, as well as arthropod ectoparasites which cause superficial wounds, could facilitate the transmission of the pathogen. It is worth noting the previous finding by IFAT of *P. salmonis* in *Caligus* sp. and *Ceratothoa gaudichadii*, 2 common parasites of cultured salmon in Chile (Garcés et al. 1994).

Mortality rates following GP exposure indicated that gills are also a route of entry and this site may be important under natural conditions. Sequential examination by IFAT of gills showed that the bacteria can penetrate the epithelium and reach the capillary vessels of the secondary lamellae. The earliest time that the bacteria were found in the capillaries was at 56 h p.i. (Table 1). In a previous report, *Piscirickettsia salmonis* was detected inside gill capillaries as early as 45 min following a 3 min immersion challenge (Smith et al. 1997) although the disease was not reproduced in this experiment. In both works the

number of bacteria observed was small and the finding of the bacteria could have been easily overlooked. In any case it seems that *P. salmonis* can reach the gill capillaries, and probably disseminate through the body, in a relative short period of time ranging from minutes to 56 h. Intact gills have been documented to be the entry site for a number of viral (Ahne 1978, Mulcahy et al. 1983, Evelyn 1996) and bacterial fish pathogens including *Vibrio anguillarum* (Nelson et al. 1985, Baudin Laurencin & Germon 1987) *Aeromonas salmonicida* (Tatner et al. 1984) and *Pasteurella piscicida* (Kawahara et al. 1989).

The low mortality obtained in fish infected by GI (2%) suggests that oral exposure is not an important route of transmission in this disease. No rickettsial organisms were detected subepithelially in the stomach serial samples and in the kidney smears following GP exposure, also indicating a failure of *Piscirickettsia salmonis* to invade the fish using this entry site. It is possible that non-specific factors such as the low pH of the stomach and/or digestive enzymes could inactivate *P. salmonis*. A fluorescent background of heterogeneous material (ca 0.5 μ m in diameter) was clearly observed in the gastric lumen, from 5 min to 1 h after IG inoculation. This antigenic material could be degraded *P. salmonis* cells. The fact that the fish were fasted prior to inoculation could have affected the results because *P. salmonis* may be protected inside fish food. Acid degradation of antigens in the stomach is a cause of poor effectiveness of oral vaccination in fish (Gorgetti et al. 1997). Johnson & Amend (1983) reported that the administration of *Yersinia ruckeri* vaccine through anal intubation of rainbow trout produced better protection than oral vaccination. Conversely, Baldwin & Newton (1993) documented penetration of *Edwardsiella ictaluri* after gastric intubation in channel catfish *Ictalurus punctatus*.

Cumulative mortality in fish infected by AI was higher than GI, reaching 24%. In the fish sampled after AI exposure, *Piscirickettsia salmonis* was detected in the kidney smears at the 3 sampling times (60 h and 7 and 14 d) and no degraded antigenic material was observed in the intestine. These findings show that the intestine is a likely entry site rather than the stomach. Therefore, ascending infections through the anal opening and/or infectious material in food that could pass the stomach without inactivation might also lead to infection of fish by the intestine in natural conditions. Nevertheless, it should be noted that both entry routes related to the alimentary canal (GI and AI) were less

efficient than the skin either intact, by SP, or through SC injection for development of piscirickettsiosis.

Recently, Almendras et al. (1997) reported a comparison of experimental infections, with *Piscirickettsia salmonis* via IP, oral (gastric intubation) and gills (instillation) in juvenile Atlantic salmon *Salmo salar*. Their cumulative mortality rates were 57, 41 and 45%, respectively. Those results are somewhat similar to the present work because they showed that gills might be important portals of entry for natural transmission of *P. salmonis*. However, their findings were dramatically different in respect to the gastric intubation. Even though Almendras et al. (1997) used a lower dose of *P. salmonis* (14.8 TCID₅₀ contained in a volume of 100 µl compared with 10⁴ TCID₅₀ contained in a volume of 200 µl used here), the mortality in their study was higher (41 versus 2%). The differences in the bacterial strain and the salmonid species used, along with the fact that the fish used by Almendras et al. (1997) had a concurrent infection with *Aeromonas salmonicida*, may partially explain these contradictory results.

In the present work, *Piscirickettsia salmonis* was detected in one case at an entry site different from the one specifically inoculated. The rickettsia was observed in a smear taken from the skin of the vicinity of the operculum at 30 min after the GP exposure. Given the low bacterial number detected (only 2 organisms were seen), it seems unlikely that this cross-infection was significant in the outcome of the experiments.

In the sequential sampling analysis by IFAT, only small amounts of bacteria were detected in the histological cross-sections of the organs (Table 1). It is possible that a lack of sensitivity of the IFAT and the thin area observed in the cross-sections caused us to overlook the bacterium.

Results of this work suggest that main entry sites of *Piscirickettsia salmonis* are through skin and gills and that the oral route may not be as important in this disease. Invasion through the intact skin is an interesting feature shown by *P. salmonis*. Further studies by electron microscopy and the searching for bacterial attachment factors and cellular receptors are required. As a preventive measure, it would be advisable to avoid ectoparasites and any other cause of skin damage to decrease the risk of piscirickettsiosis in cultured salmon. Finally, research to find drugs that prevent the entry of *P. salmonis* to its host and/or investigation orientated to increasing local immunity at skin and gill levels seems to be interesting to explore.

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A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins

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Abstract

We report here the protective effect against piscirickettsiosis elicited in fish by a mixture of recombinant proteins. A comparative genomics strategy was used on a genomic library of *Piscirickettsia salmonis* in order to select optimal candidates for a recombinant subunit vaccine to protect fish from rickettsial septicaemia (SRS). Based on this information, 15 *P. salmonis* ORFs encoding heat shock proteins, virulence factors, membrane bound and other surface exposed antigens, were isolated and expressed. Seven of the most promising antigens were formulated in three mixtures (V1–V3) containing two or three recombinant proteins each and injected into salmon to test their protective efficacy. Two of the three formulations (V1, V2) elicited a strong protective response in a challenge against the pathogen, which was coincident with the humoral response against the corresponding recombinant proteins present in each formulation. V1, formulated with recombinant chaperonines Hsp60, Hsp70 and flagellar protein FlgG of *P. salmonis* achieved the highest level of protection with a relative percent survival (RPS) of 95%.

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1. Introduction

Piscirickettsia salmonis is the etiological agent of the salmonid rickettsial septicaemia (SRS) or piscirickettsiosis. This bacterium, isolated in 1989 from a moribund coho salmon from a saltwater net pen site in the south of Chile, was the first Rickettsia-like organism recognized as a fish pathogen [1]. Since then, the disease has also been reported to affect Atlantic salmon, the main salmonid species cultured in Chile, as well as rainbow trout and other farmed salmon species. Outbreaks of SRS have also emerged among farm-raised salmon in Canada, Norway and Ireland, however, mortalities have not been as high as those in Chile [2].

The pathogen has also been isolated from sea bass in California and *Piscirickettsia*-like organisms have been identified in Hawaiian tilapia and several other fish species [3], indicating that the disease is not only confined to salmonids.

The pathogen is a gram-negative, obligate intracellular bacterium. It is pleiomorphic, predominantly coccoid in shape and ranging in diameter from 0.5 to 1.5 μm . Molecular phylogenetic analysis based on sequencing of the 16S rRNA gene placed *P. salmonis* in a new family of *Piscirickettsiaceae* within the class of γ -proteobacteria, most closely related to *Coxiella*, *Francisella* and *Legionella* [4]. *P. salmonis* produces a systemic infection in fish targeting predominantly the kidney, liver, spleen, intestine, brain, ovary and gills. Fish begin to die 6–12 weeks after their transfer to seawater net pens in fall and spring. The Chilean aquaculture industry attributes annual losses of US\$ 150 million to SRS [5], having

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an important effect on the economy of a country positioned as the second largest exporter of salmon and trout after Norway.

Although *P. salmonis* is sensitive in vitro to many antibiotics commonly used to control other infectious diseases in fish, infected salmonids respond poorly to this treatment, due perhaps to an insufficient concentration of antibiotics within the host cell to kill the pathogen [2]. The lack of effective treatments to control piscirickettsiosis has emphasized the need to develop techniques for disease prevention. Management of the disease is based on several husbandry practices including the application of immunostimulants of unproven efficacy and the control of vertical transmission by an expensive selection procedure during reproduction. Although vaccines made of inactivated bacteria have been successfully used to control certain bacterial disease in fish [6], preparations based on *P. salmonis* bacterins have not yielded significant protection against SRS [5,7]. This might be related to the loss of important surface antigens during both, culture of the pathogen in animal cell lines, as well as in the inactivation process. A recombinant subunit vaccine is an interesting alternative. Since its first application [8], recombinant DNA technology has been considered as a valuable technology for development of vaccines against many human and animal pathogens, including *Rickettsiae* [9], a class of intracellular bacteria related to *Piscirickettsia*. In addition, the potential use of recombinant vaccines in aquaculture has been discussed extensively [10,11]. Recently a recombinant vaccine has been introduced into the market. This product is based on the 17 kDa OspA outer surface lipoprotein from *P. salmonis* fused in tandem to T cell epitopes from tetanus toxin and measles virus. This preparation attained an 83% RPS when tested in coho salmon [7]. However, there is a need for further improvement specially regarding the creation of multivalency as a mean to insure wider protection against emerging isolates.

The present work describes the use of a predictive genomics strategy to select as vaccine targets *P. salmonis* proteins previously identified as virulence factors and protective antigens in other microorganisms. We postulate that the presence of various recombinant antigens in a treatment might improve the efficacy of the vaccine. Moreover, the inclusion of antigens conserved through species could have a cross-protective effect among different bacterial pathogens. Our efforts have been directed to express recombinant heat shock proteins and surface antigens of *P. salmonis* as antigens for an effective vaccine. We report here the protective effect against piscirickettsiosis elicited in fish by a mixture of recombinant proteins.

2. Materials and methods

2.1. Cell culture

The Chinook salmon embryo cell line CHSE-214 (ATCC 1681) was cultured in complete MEM (Gibco BRL) supple-

mented with non-essential amino acids, glutamine and 5% fetal bovine serum (GIBCO BRL), in T175 flasks at 16 °C.

2.2. Bacterial strains and plasmids

Escherichia coli strains NovaBlue and BL21(DE3), used for cloning and expression, respectively, were obtained from Novagen. *P. salmonis* Bios-007 was isolated in 1995 from the liver of a sick fish obtained at the location of Calbuco, in the South of Chile. To grow *P. salmonis*, frozen inoculates of about 1×10^8 bacteria/mL, were brought to room temperature, added to flasks containing confluent CHSE-214 cells and incubated overnight at 16 °C. The medium was then replaced by fresh complete MEM supplemented with non-essential amino acids, glutamine and FBS 5% and cultured for 10–14 days at 16 °C. Periodic checks of the degree of cytolysis were performed. Cultures were considered ready for harvesting when nearly 100% of the cells were lysed. Cells adhered to the flask walls were scraped, centrifuged twice at $150 \times g$ at 10 °C and the second supernatant saved as the semipurified fraction of *P. salmonis*. Further purification was performed according to Jamett et al. [12].

The plasmids pET32a (Novagen) and pGEMT (Promega) were propagated in NovaBlue cells in medium LB with 100 µg/mL ampicillin at 37 °C. *E. coli* BL21(DE3) cells transformed by pET32a were grown in LB with 100 µg/mL ampicillin.

2.3. Cloning of *P. salmonis* antigen coding regions

Genomic DNA was extracted from *P. salmonis* as described previously [13]. Predicted coding regions of selected antigens of *P. salmonis* were isolated by PCR amplification using specific primers (Table 1) based on the sequence information from the *P. salmonis* genomic library obtained in our laboratory. Amplified products were purified using a kit from Qiagen, ligated to pGEMT and used to transform NovaBlue competent cells. Positive clones were selected by blue/white screening using lacZ α -complementation.

2.4. DNA analysis and sequencing

Plasmid DNA was purified using a kit from Qiagen. DNA samples and restriction endonuclease digests were analyzed by electrophoresis in agarose gels. The pGEMT constructs were sequenced with the Big Dye Terminator Cycle Sequencing V.2.0 kit (Applied Biosystem Inc.) based on the procedure of Sanger et al. [14] using a 310 Genetic Analyzer (Applied Biosystem Inc.).

2.5. Production of recombinant proteins in *E. coli*

The coding regions of the selected genes were amplified by PCR using specific primers with restriction endonuclease sites at their 5' ends. The amplified coding regions were

Table 1
Oligonucleotides used as primers

Gene	Forward primer	Reverse primer
Hsp10	5'-ggcgaattcatgaaaatccgtccattacat-3'	5'-cggtctcgaggaaataatcttcaacgactgc-3'
Hsp16	5'-cgcgatatcatgagtcatttaattatccc-3'	5'-gccctcgagctatgccattttttatctacta-3'
Hsp60	5'-gacggatccggagataaagaatgtcagca-3'	5'-tatgaattcttaaccgccatgccacccat-3'
Hsp70	5'-tatgaattcatggctgaaattattggttg-3'	5'-gtactcgagctaaactctcaaaactcagcatc-3'
MltB	5'-gacgaattcatgagacgatcttattggcta-3'	5'-gacctcgagttttaagagccttttgagtg-3'
Slt70	5'-caggaattcgacataatgccatactacatt-3'	5'-cagctcgagttaaacacgcctaattccagcatt-3'
TbpB	5'-cttggatccatgaaactaccatagcttgattgg-3'	5'-cttaagcttctcatttaattgagcagc-3'
31 kDa protein	5'-caggaattcgttattggcagcaccacat-3'	5'-gctctcgagatgccttagtttaaccccg-3'
VacB	5'-ggaagatatcatggtaaaaaagaagacaacaag-3'	5'-ggttggatccctaagctcttttgatgttcattt-3'
Omp27 (kDa)	5'-cagggatccgccatgagaagcaaacacc-3'	5'-caggaattcatgggtgagtttctgtg-3'
Mp13 (kDa)	5'-ctcgatccctaattatgcagttttctgtg-3'	5'-gacgaattcccaagtattattgtatcagtagt-3'
FlgF	5'-gcagga tccgtgatcatggacatggaatt-3'	5'-tgcgaattcttaattgcataatgcgtaccg-3'
FlgG	5'-cagggatccaggattatgattccagcattat -3'	5'-ctggaatccctgattatcatgctgtgatttaagaa-3'
FlgH	5'-gacggatccaatattaagatgaggagtttatgg-3'	5'-ctggaattcctagaatggccatatcacact-3'
FlaA	5'-cagggatccatggaaggaagggcgactga-3'	5'-cacgaattcctagataagtgatagtagcg-3'

cloned into the expression vector pET32a in frame with the thioredoxin gene of *E. coli* and a histidine-tag domain. *E. coli* BL21(DE3) competent cells were transformed with the constructs and expression of recombinants was induced by incubation in LB-ampicillin supplemented with 1 mM IPTG. Recombinant proteins used for immune analysis were purified by a Ni-agarose column (Qiagen). For vaccine formulations, recombinant proteins were used as semipurified preparations either as inclusion bodies or soluble protein fractions obtained by centrifugation of sonicated bacteria at $20,000 \times g$ for 15 min. Protein concentration was measured using the Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli [15].

2.6. Monoclonal antibody production

Two-month-old female BALB/c mice were injected intraperitoneally at 3 weeks intervals with three doses of 50 μ g of purified recombinant proteins diluted in PBS and emulsified with Freund adjuvant. Ten days after the last injection, the animals were bled from the tail to obtain serum. The humoral response against the recombinant proteins was determined by an ELISA test [12]. To produce hybridoma, spleen cells from the immunized mice were isolated and fused with NS0/2 mouse myeloma cells [16].

2.7. Determination of the lethal dose 50 (LD50)

Two hundred and fifty fish (*Salmo salar*) with an average weight of 18 g were tagged and kept 2 weeks in fresh water under controlled conditions to recover before being challenged. The fish were randomly distributed in 10 groups of 20 fish. Fish from each group were injected intraperitoneally with a 200 μ L suspension of increasing doses of *P. salmonis* from 1×10^2 to 1×10^8 bacteria titrated as described previously [17]. Doses were prepared by serial dilutions of semipurified *P. salmonis* corresponding to the eleventh pas-

sage of isolate Bios-007. A control group, injected with the saline solution used to resuspend the pathogen, was also included. The injected fish of each group were distributed in two tanks of 1000 L (10 fish of each dose per tank) and maintained at 13 °C under controlled conditions of oxygenation, feeding and water flow until mortalities ended. Cumulative mortalities at each dose were plotted and the LD50 was determined [18]. SRS was confirmed by histopathological analysis of dead fish.

2.8. Recombinant vaccine trial

Three experimental formulations containing two or three recombinant proteins were prepared. Each mixture was emulsified with one volume of incomplete Freund adjuvant to obtain a 1:1 oil in water preparation with a final concentration of 50 μ g/mL of each protein. *Salmo salar* with an average weight of 18 g were tagged for group identification. Three groups of 104 fish each were injected intraperitoneally with 0.2 mL of each vaccine preparation that contained 10 μ g of each recombinant protein. The vaccinated fish were randomly distributed in eight tanks with 13 fish of each group per tank. Control fish injected with saline and incomplete adjuvant were also included in the same tanks. Salmon were held at 13 °C under controlled conditions of oxygenation, feeding and water flow for 7 weeks post-vaccination (624 degree days).

For challenge, control and vaccinated fish were injected intraperitoneally with 0.2 mL of *P. salmonis*. The fish from four tanks (208 fish) were injected with a dose of *P. salmonis* equivalent to $2 \times$ LD50 and an identical number of fish from other four tanks were injected with a dose of bacteria equivalent to $8 \times$ LD50. Fish of each tank injected with $8 \times$ LD50 were transferred to each tank of fish injected with $2 \times$ LD50 in order to have four replicas (26 fish of each group per tank) each under the same feeding and environmental conditions.

2.9. Calculation of the relative percent survival (RPS)

The protection elicited by the vaccine formulations was determined by comparing the cumulative mortality of treated and control groups. The RPS, was calculated according to the equation: $RPS = [1 - (\% \text{ mortality of test group} / \% \text{ mortality of control group})] \times 100$ [19].

2.10. Western blot analysis

Recombinant proteins were separated by PAGE-SDS gel electrophoresis and transferred to nitrocellulose. Total protein (30–40 µg) obtained from *P. salmonis* were analyzed with a proper dilution of the monoclonal antibodies and developed with an anti-mouse IgG conjugated with alkaline phosphatase. The monoclonal antibodies used against FlgG, FlgF, FlaA, Omp27, Hsp60, Hsp70, VacB, 31 kDa protein and TbpB were 4H8/G8, 7H11/H9, 5A4/G12, 4G1/00, 5EIO/G7, 3D5/A11, 5F7/E2, 5611/H11 and 7E1/D6, respectively. To analyze the immune response of vaccinated salmon, nitrocellulose membranes containing 1 µg of the corresponding recombinant protein were incubated with a 1:200 dilution of salmon serum. Blots were then incubated with an anti-salmon IgM monoclonal antibody and developed with an anti-mouse IgG conjugated with alkaline phosphatase.

3. Results

3.1. Identification of *P. salmonis* proteins as potential vaccine candidates

About 80% of the genome of *P. salmonis* has been sequenced in our laboratory. Approximately 20,000 individual sequences were obtained in both directions and assembled in 2143 contigs from a random library of genomic fragments. Although the genomic information is not complete, analysis

of the contig sequences by comparison with other bacterial genomes has permitted the prediction of nearly 1500 genes of which 90% could be assigned to a known function.

Vaccine candidate were selected by searching for *P. salmonis* genes that encode proteins with sequence similarity to virulence factors involved in host-pathogen interactions or immunoreactive antigens that are secreted or located at the surface of other known pathogens. More than 40 genes were selected by these criteria, of which 15 were selected for further analysis (Table 2). These include the virulence factor VacB [20]; structural components of a putative flagellar structure such as FlgG, FlgH, FlgF and FlaA [21,22]; members of the heat shock family Hsp60, Hsp70, Hsp10 and Hsp16, which are known to be strong immunogenic determinants [23]; the membrane proteins Mp13 [24], Omp27 [25], 31 kDa [26], the transferrin binding protein TbpB and the membrane lytic transglycosylase MltB [27] and its soluble counterpart Slt70 [28].

3.2. Cloning and expression of recombinant *P. salmonis* proteins

In order to isolate the complete coding region of the 15 selected genes by PCR, specific primers were designed based on the genomic sequences. Some open reading frames (ORFs) were completely contained within a single contig and they were easily isolated and sequenced. Other ORFs were contained in two or more contigs and were isolated by PCR with primers designed according to the sequence flanking the missing regions. The conserved organization of some genes in clusters was particularly useful to isolate genes whose sequences were only partially represented in a single contig. Using these strategies, the 15 chosen ORFs were isolated, cloned into pGEMT and their sequences confirmed. The coding regions of each gene were then subcloned in frame with the *E. coli* thioredoxin (*Trx*) coding region present in the prokaryotic expression vector pET32a. Fusion proteins were

Table 2
Selected vaccine candidates

Gene	Protein	Basis for selection
Hsp10	Heat shock protein	Cellular and humoral response [23]
Hsp16	Heat shock protein	Cellular and humoral response [23]
Hsp60	Heat shock protein	Cellular and humoral response [23]
Hsp70	Heat shock protein	Cellular and humoral response [23]
MltB	Periplasmic membrane lytic transglycosylase anchored to the outer membrane	Strong cellular and humoral response in <i>N. meningitides</i> [27]
Slt70	Periplasmic soluble lytic transglycosylase	Highly expressed in the periplasm [28]
TbpB	Transferrin binding protein localized in the outer membrane	Strong cellular and humoral response in <i>N. meningitides</i> [27]
31 kDa protein	Outer membrane protein	Immunogenic protein in <i>B. abortus</i> [26]
VacB	Cytoplasmic virulence factor B	Virulence factor [20]
Omp27 kDa	Outer membrane protein	Extracellular antigen [25]
Mp13 kDa	Membrane protein	Defense against <i>F. tularensis</i> [24]
FlgF	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgG	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgH	L ring of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlaA	C-terminus of flagellin, subunit of the extracellular flagellar filament	Flagellum is highly immunogenic [21,22]

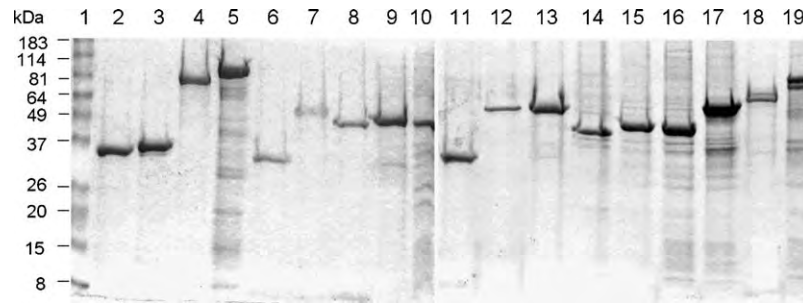


Fig. 1. SDS-PAGE analysis of purified *P. salmonis* recombinant proteins expressed in *E. coli*: (1) molecular weight markers; (2) Trx-Hsp10; (3) Trx-Hsp16; (4) Trx-Hsp60; (5) Trx-Hsp70; (6) Trx-Omp-C; (7) Trx-FlaA-C; (8) Trx-FlgF; (9) Trx-FlgG; (10) Trx-FlgH; (11) Trx-Mp13; (12) Trx-31 kDa protein; (13) Trx-TbpB-N; (14) Trx-MltB-N; (15) Trx-MltB-C; (16) Trx-Slt70-N; (17) Trx-Slt70-C; (18) Trx-VacB-N; (19) Trx-VacB-C. N and C refer to amino and carboxyl domains, respectively.

expressed in BL21(DE3) cultures under the control of T7Lac promoter upon induction with IPTG. The results are shown in Fig. 1. Most recombinant proteins were expressed as inclusion bodies with the exception of Trx-Hsp10, Trx-Hsp16 and Trx-Hsp70, which were soluble [29,30]. The ORFs of TbpB, VacB, Omp27, Slt70, FlaA and MltB were expressed either partially or in two halves [31].

3.3. Expression of the selected proteins in *P. salmonis*

It was of interest to study if the antigen candidates are expressed by the pathogen during infection. Therefore, extracts of *P. salmonis* growing in CHSE-214 cells were analyzed by Western blot for the presence of the native proteins. Specific monoclonal antibodies were used to detect the presence of the selected proteins in these extracts. As seen in Fig. 2, strong signals corresponding to Hsp60 and Hsp70 were found in extracts of *P. salmonis*. Less abundant, but clearly detected, are the proteins 31 KDa, TbpB and VacB. Although flagellar proteins FlgF, FlgG, flagellin

and Omp27 were shown to be highly immunogenic in mice, monoclonal antibodies obtained against these recombinant proteins did not detect their presence in the bacterial extracts (results not shown). The level of expression of Slt70, Hsp10, Hsp16, FlgH, Mp13 and MltB in *P. salmonis* extracts was not analyzed due to the lack of monoclonal antibodies. Sera of mice immunized with these proteins were not used to analyze expression in bacterial extracts since they also presented reactivity against the thioredoxin moiety of the fusion proteins.

3.4. Recombinant vaccine trial

To measure the protective effect elicited by the recombinant proteins, formulations containing two or three of the most promising candidate proteins were prepared to be tested in a challenge with *P. salmonis*. Analysis of these mixtures by gel electrophoresis is shown in Fig. 3. The first group

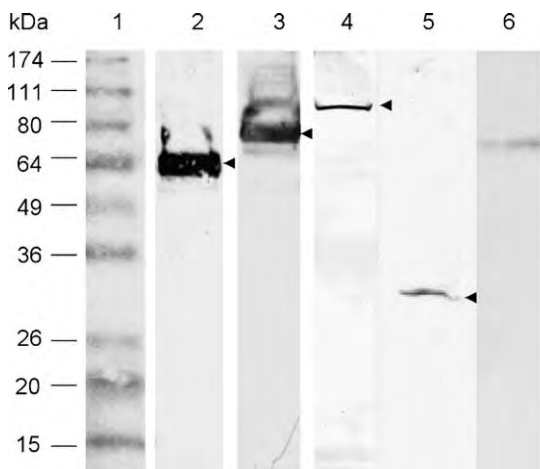


Fig. 2. Western blot analysis of antigens present in *P. salmonis*. Gel was loaded with 30–40 µg of total *P. salmonis* protein and the blotted membranes were analyzed with a 1:200 dilution of each monoclonal antibody: (1) molecular weight markers; (2–6) monoclonal antibody against Hsp60, Hsp70, VacB, 31 kDa protein and TbpB, respectively.

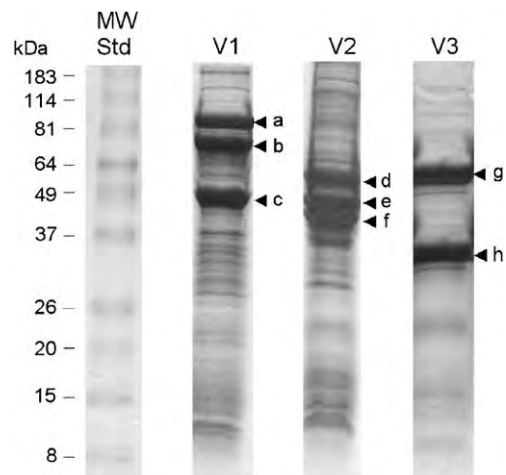


Fig. 3. SDS-PAGE analysis of recombinant vaccine formulations. Oil in water formulations containing 10 µg of each recombinant protein were used to immunize fish. MW Std: molecular weight markers; V1: formulation containing partially purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c). V2: formulation containing partially purified Trx-TbpB-N (d), Trx-MltB-C (e), Trx-MltB-N (f). V3: formulation containing partially purified Trx-FlaA-C (g) and Trx-Omp-C (h).

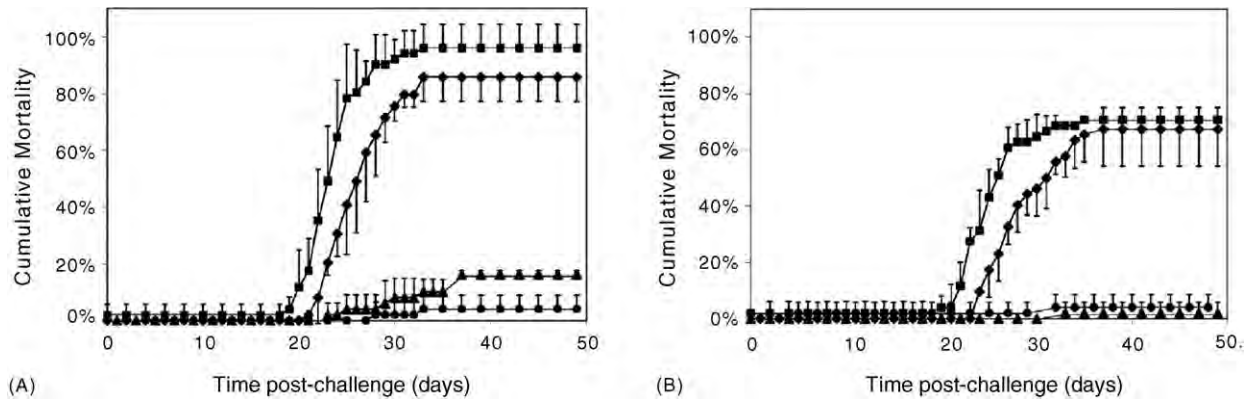


Fig. 4. Protection of recombinant vaccine against SRS in Atlantic salmon. (A) Cumulative mortality of control and immunized fish challenged with a dose of $8 \times \text{LD}_{50}$ of *P. salmonis*. (B) Cumulative mortality of control and immunized fish challenged with a dose of $2 \times \text{LD}_{50}$ of *P. salmonis*. (■) Control fish immunized with adjuvant; (●) fish immunized with formulation V1; (▲) fish immunized with formulation V2; (◆) fish immunized with formulation V3. The curves represent the average cumulative mortalities of four tanks of fish and the standard deviation of each value is indicated as a bar.

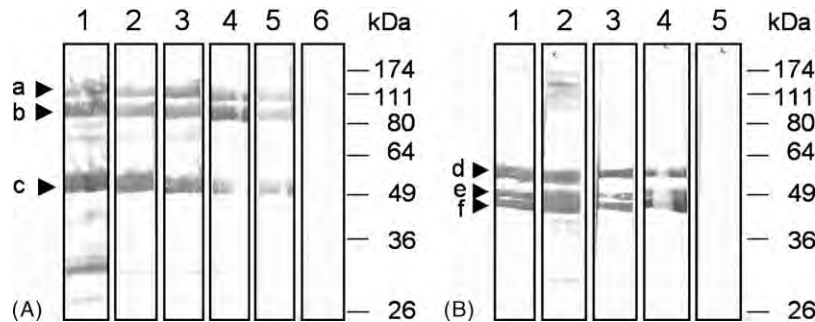


Fig. 5. Western blot analysis of serum obtained from salmon immunized with recombinant formulations V1 and V2. (A) Blots containing purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c) tested with serum of fish immunized with V1 formulation (lanes 1–5) and serum of non-immunized fish (lane 6). (B) Blots containing purified Trx-TbpB-N (d), Trx-MltB-C (e) and Trx-MltB-N (f) tested with serum of fish immunized with V2 formulation (lanes 1–4) and serum of non-immunized fish (lane 5).

(V1) is composed of Hsp60, Hsp70 and FlgG. The second group (V2) is composed of the amino terminal half of TbpB and the carboxyl and amino terminal halves of MltB. The third group (V3) is composed of the recombinant carboxyl portions of Omp27 and FlaA. Oil in water emulsions of the three recombinant preparations were injected intraperitoneally into Atlantic salmon of approximately 18 g. A saline-adjuvant control was also included in the trial.

The result of the challenge experiment with a dose equivalent to $8 \times \text{LD}_{50}$ of *P. salmonis* is shown in Fig. 4A. As expected, an increasing mortality was observed in the control group. This effect began at day 21 post-challenge and reached a cumulative mortality of 96% at day 49 post-challenge. In the fish vaccinated with V3, the formulation elicited only a mild protection, with a RPS of 10.4%. Fish vaccinated with V2 exhibited a significant protective response with a RPS of 84.4%. The highest protective response was achieved with V1, reaching a RPS of 95.8%. These results represent the average mortality of each group of fish in the four tanks, which behaved as consistent replica of the trial with minor deviations (Fig. 4). As expected, the fish challenged with a *P. salmonis* dose equivalent to $2 \times \text{LD}_{50}$ achieved lower cumu-

lative mortalities (Fig. 4B), and the protective response of the V1 group (RPS 94.4%) was similar and consistent with the observed protection against the $8 \times \text{LD}_{50}$ dose of *P. salmonis*.

3.5. Immune response of salmonids against recombinant proteins

Sera obtained at the end of the trial from four or five surviving fish of each of the V1 and V2 vaccinated fish was used to analyze the immune response against the recombinant component of each formulation. Serum obtained from a non-vaccinated and non-challenged fish was used as a negative control. A measurable immunoreaction against the recombinant proteins of V1 and V2 was detected by Western blot (Fig. 5), which is coincident with the ELISA immune reaction previously reported [30,31]. The specificity of the immune reaction elicited by the sera from V1 vaccinated fish was confirmed by the positive reaction against Hsp60, Hsp70 and FlgG previously excised of their thioredoxin fusion peptide (results not shown). Interestingly, the humoral response of V1-vaccinated salmon against recombinant proteins Hsp60, Hsp70 and FlgG was still high 8 months post-vaccination

(2800 degree days) as indicated by the immune reaction of their sera in a Western blot (data not shown).

4. Discussion

Prevention strategies must consider the need for a good cellular immune response to protect against the intracellular pathogen *P. salmonis*. In this respect, the most promising strategies are DNA vaccines or recombinant protein vaccines. In a first attempt using DNA vaccination against *P. salmonis*, our laboratory utilized the expression library technology to study the protection of coho salmon to the infection with *P. salmonis* with very limited success [32]. In the present work, we have concentrated our efforts on the recombinant protein subunit approach. In the case of bacterial pathogens, the selection of key proteins is essential for this strategy to succeed. We focused on molecules previously identified as virulence factors and protective antigens in other bacteria and associated with the bacterial surface. The available partial sequence of the *P. salmonis* genome permitted us to isolate and express genes to be tested as recombinant protein vaccines. Of the fifteen recombinant proteins, seven of them included in this trial were selected according to the protection conferred against other pathogens in a variety of organisms [22,23,25,27], their location in the bacterial cell and the immune response elicited in mice. The level of expression of these proteins in bacteria was also a factor considered. Two of the formulations (V1 and V2) elicited a strong protection against *P. salmonis*.

In our trials, the highest protective response (95% RPS) against *P. salmonis* was elicited by formulation V1, which contains the FlgG subunit of the basal flagellar structure and chaperonins Hsp60 and Hsp70. This result suggests that the use of more than one recombinant antigen might potentiate the immune response. However, given the varying degrees of protection achieved with the different preparations, the proper selection of antigens appears as crucial for successful protection. The efficacy of formulation V1 was confirmed by three independent challenges of Atlantic salmon, eliciting relative percent survivals of 94%, 88% and 91% (data not shown). This high level of protection in Atlantic salmon is particularly important since this is the main salmon species farmed in Chile. This level of protection was also confirmed in coho salmon by an independent research effort performed by investigators from the company that licensed this technology, who obtained an RPS of 94.5% when vaccinated coho salmon were challenged with *P. salmonis* after 1000 degree days (personal communication).

Protein FlgG is a structural component of the flagella basal body [33]; a structure very similar to the needle complex of the system III involved in the secretion of virulence factors and is present in flagellated and nonflagellated pathogens. The components of the flagellar basal body are synthesized by the *flg* operon. Recently it has been reported that they play an important role in the virulence and adhesion of the

pathogen *Vibrio vulnificus* [34]. The expression of FlgG in *P. salmonis* has been indirectly proven by the specific immune reaction against FlgG of a serum from a rabbit immunized with *P. salmonis*, and by the detection of transcripts encoding this protein [Wilhelm et al., manuscript in preparation]. The efficacy of vaccine V1 is also in agreement with the protective effect elicited by Hsp60 and Hsp70 in other animal models [23]. The strong immune reaction observed with our monoclonal antibodies against Hsp60 and Hsp70 in extracts from *P. salmonis* can be correlated with the abundance of these proteins during infection in other intracellular pathogens [35]. Additionally, Hsp60 and Hsp70 have been localized on the periplasm as well as on the bacterial surface or as extracellular secreted proteins during host infection [36–38]. The surface location of the Hsp proteins as well as other properties of these molecules have been suggested to play a significant role in mediating attachment, invasion of host cells and immune modulatory activities [39,40] that could explain the strong protection elicited when the Hsp are included in vaccines. It has been proposed that the high conservation of Hsp among various microbial pathogens generates an immunologic memory for Hsp cross-reactive determinants during life due to frequent restimulation by subsequent encounters with microbes [23]. Consistent with this notion, the use of Hsp in a vaccine has the advantage of eliciting an immune response to conserved determinants shared by different bacteria, preventing colonization of the host by other microbial pathogens. Although a concern could exist regarding the use of these conserved proteins in vaccines due to the risk of an autoimmune response, the observed cross recognition of host Hsp by reactive T cells has been proposed to play a role in autoimmune processes only during chronic inflammation of the host [23]. In fact, we detected no cross-reaction with Hsp of the salmon embryonic CHSE-214 cell line with the antibodies against Hsp from *P. salmonis* (not shown). Similarly, antibodies against bacterial Hsp60 raised in mice cross-react with Hsp60 homologues of other prokaryotes, but not with the murine Hsp homologues [23].

The protection elicited against *P. salmonis* by V1 is slightly better than the protection of coho salmon reported previously for a recombinant vaccine composed of the 17 kDa antigen OspA [7]. In that report, an 83% efficacy was obtained when a quimeric protein of OspA and two T cell epitopes (TCE's) from tetanus toxin and measles virus was used, while recombinant OspA alone had a protective effect with a RPS of 30.2%. The increased efficacy of the OspA recombinant vaccine conferred by these TCE's, demonstrates for the first time the immunostimulatory effect of mammalian TCE's on the salmon immune system. One of the advantages of including Hsp in the vaccine described in this paper is the natural adjuvant effect of Hsp60 by mediating a Th1 type immune response [40] and thus favouring a cellular immune response without the need for additional TCE's. Moreover, the continuous stimulation of the immune response by cross-reactive determinants among the conserved bacterial Hsp may be an additional benefit of this vaccine because it might generate a

more lasting immunologic memory. Although long-term protection was not measured in adult fish in this study, reactive antibodies against the recombinant proteins present in formulation V1 were detected in sera of fish tested at 8 months post-vaccination. Additional analysis of cellular immune response should be considered to further explain the protective effect of the vaccine against this intracellular pathogen, which will be the focus of future research. In this regard, a protective role of LPS and some *E. coli* antigenic proteins present in these partially purified recombinant mixtures should not be ruled out. Indeed, an efficacious induction of cellular immunity has been demonstrated in the differentiation of helper T cells to a Th1 phenotype by *E. coli* LPS in vivo [41]. This evidence and the demonstration that salmon macrophages infected with *P. salmonis* express immune genes corresponding to those expressed by LPS stimulated human macrophages [42] strongly argues for a positive protective effect due to the inclusion of *E. coli* LPS in the recombinant vaccine reported here.

Formulation V2, which consists of the membrane transglycosylase and transferrin binding protein B accomplished a strong protection eliciting 85% RPS. The protective potential of these two proteins is coincident with their immunoreactivity in *Salmo salar* [31] and their capacity to induce a bactericidal activity [27,43]. Although only TbpB expression was analyzed, both proteins are significant determinants of virulence and are highly expressed by bacteria during infection [44,45]. An increase in the expression of bacterial transferrin receptors is thought to play a critical role in the infection process allowing the bacterium to compete effectively with its host for limiting iron. In addition, it has recently been shown an increased synthesis of transferrin in salmon macrophages infected with *P. salmonis*. This phenomenon has been proposed as an alternative mechanism of defence against intracellular pathogens [42]. *P. salmonis* may respond by an increased synthesis of transferrin receptors to overcome the lack of iron. These data suggest that this family of receptors is one of the most promising vaccine candidates against many pathogens [27,43,46].

Although the *E. coli* thioredoxin fusion peptide may have caused an immune reaction in mice and salmon, this protein is apparently not involved in the protection elicited by the formulations V1 and V2, because formulation V3 (that had a minor protective effect against the pathogen) also contained thioredoxin as a fusion peptide to the FlaA or Omp27 proteins. OmpA and FlaA were chosen as antigens in formulation V3 due to their high immunogenicity in mice (not shown). The lack of protection of formulation V3 in fish could be explained by the fact that only partial fragments of protein Omp27 and flagellin A were included in this mixture. Also, neither of these two proteins has been detected in *P. salmonis* with the monoclonal antibodies developed here. Our failure to detect expression of these proteins could be due to the culture conditions that may affect the expression of these antigens in vitro. In this regard, it is interesting to comment that although the pathogen has been considered non-motile, more than 37 flagellar genes exist in our genomic data [Wilhelm et al.,

manuscript in preparation]. Detection of transcripts encoding for some of the subunits of the flagellar structure suggests the possibility that flagella may be synthesized under certain conditions. This may influence transmission from one fish to another during the short extra-cellular stage of *P. salmonis* in seawater.

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Review

Piscirickettsiosis and piscirickettsiosis-like infections in fish: a review

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Abstract

Piscirickettsia salmonis was the first “rickettsia-like” bacteria to be recognized as a pathogenic agent of fish. Since the first reports of *piscirickettsiosis* emerged from Chile in the late 1980s, *Piscirickettsia*-like bacteria have been recognized with increasing frequency in a variety of fish species, from both fresh and saltwaters around the world. Although the first reported incidents of *Piscirickettsia* were in salmonids, *Piscirickettsia*-like bacteria are now being frequently associated with disease syndromes in non-salmonid fish. Mortalities have occurred in white seabass (*Atactoscion nobilis*), black seabass (*Dicentrarchus* sp.), tilapia (*Oreochromis*, *Tilapia* and *Sarotherodon* spp.) and blue-eyed plecostomus (*Panaque suttoni*). Piscirickettsiosis and piscirickettsiosis-like diseases have affected aquaculture productivity, profitability, the species of fish compatible with commercial rearing, and transportation of fish from site to site. Piscirickettsiosis and syndromes caused by similar bacteria are an emerging disease complex that will increasingly inhibit fish production. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Piscirickettsiosis; Rickettsia-like; *Piscirickettsia salmonis*

During the last decade members of a group of fastidious, obligate, intracellular bacteria (rickettsia-like) are being increasingly recognized as important fish pathogens. These bacteria occur globally in a variety of fresh and saltwater fish species. In April 1996 at a US congressional briefing on new and emerging infectious diseases, obligate intracellular bacteria the rickettsia-like organisms (RLOs) were identified as emerging infectious disease causing agents of fish.

The earliest report of RLOs in fish was by Mohamed (1939), in diseased puffers (*Tetrodon fahaka*) taken from the Nile river in Egypt. As no cultures were done, the report

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Table 1

Piscirickettsia-like organisms in fish from 1939–2001

Fish species	Location	Citation
Fokaka (<i>T. fahaka</i>)	Egypt	Mohamed (1939)
Dragonet (<i>C. lyra</i>)	Wales	Davies (1986)
Tilapia		
<i>Oreochromis</i> and <i>Tilapia</i> sp.	Taiwan	Chern and Chao (1994), Chen et al. (1994)
<i>Tilapia nilotica</i>	Japan	Wada et al. (1995)
<i>S. melanotheron</i>	Hawaii	Unpublished data (1994)
Unknown spp.	Jamaica	Unpublished data (2000)
	Indonesia	Unpublished data (2000)
	California	Unpublished data (2001)
	Florida	Unpublished data (2001)
Cichlid (<i>C. managuense</i>)	Taiwan	Chern and Chao (1994)
Blue-eyed plecostomus (<i>P. suttoni</i>)	Columbia	Khoo et al. (1995)
Black seabass (<i>D. labrax</i>)	France	Comps et al. (1996)
	Greece	Athanassopoulou et al. (1999)
White seabass (<i>A. nobilis</i>)	California	Chen et al. (2000)
Grouper (<i>E. melanostigma</i>)	Taiwan	Chen et al. (2001)
Atlantic salmon (<i>S. salar</i>)	Chile	Garcès et al. (1991)
		Cvitanich et al. (1991)
		Fryer et al. (1992)
	Canada	Jones et al. (1998)
Salmon and trout	Germany	Ozel and Schwanz-Pfützner (1993)
<i>Oncorhynchus</i> spp.	Chile	numerous reports 1980s–90s
	Canada	Evelyn (1992)

was based on the observation of the bacteria within stained tissue cells. Ozel and Schwanz-Pfützner (1975) observed an intracellular bacterium in cultured rainbow trout gonad (RTG-2) cells during tests of rainbow trout (*Oncorhynchus mykiss*) for Egtved virus (viral hemorrhagic septicemia virus, VHS). The next report of RLOs in fish was by Davies (1986) who observed a coccoid RLO found in membrane-bound vacuoles in lymphocytes of dragonets (*Callionymus lyra* L.) from the coastal waters of Wales (Table 1).

Bacteria endemic in an environment usually only cause sporadic disease but can induce an epizootic in the close confines present in aquaculture facility raceways and net pens. New species introduced into an area often lack the immune defense mechanisms endemic fish species have developed through time. Stress induced on the individual animals in the high population densities present in aquaculture practices can leave an animal immune deficient. The close contact, reduced immune response or lack of a primed immune response can leave a fish population susceptible to disease. An example of this is the emergence of piscirickettsiosis, also known as salmon rickettsia syndrome (SRS) in Chile. The syndrome has altered the salmonid culture in Chile, causing a shift from the more commercially desirable coho salmon to the less desirable but more piscirickettsiosis resistant Atlantic salmon as the primary cultivated species (P. Smith, University of Chile, Santiago, personal communication). In 1989, recurring piscirickettsiosis epizootics resulted in extensive mortality (90%) of coho salmon (*Oncorhynchus kisutch*) reared in seawater net pens in the near-shore waters of southern Chile (Branson and Nieto

Diaz-Munoz, 1991; Cvitanich et al., 1991). *Piscirickettsia salmonis* gen. nov., sp. nov. (Fryer et al., 1992), a previously unknown obligate intracellular pathogen was isolated, on the salmon cell line CHSE 214, from these diseased fish and demonstrated to be the etiologic agent.

1. Piscirickettsiosis

The initial description of piscirickettsiosis was in coho salmon in saltwater (Fryer et al., 1990; Cvitanich et al., 1991). However, other salmonid species reared in the area (chinook salmon, *Oncorhynchus tshawytscha*; masu salmon, *Oncorhynchus masou*; rainbow trout, *O. mykiss*; Atlantic salmon, *Salmo salar*) also experienced mortality from piscirickettsiosis in the saltwater environments (Fryer et al., 1996; Fryer and Lannan, 1996). Subsequent work has indicated that the agent and its associated disease may also occur in fresh water raised salmonids (Bravo, 1994; Gaggero et al., 1995). After the initial description in Chile, *P. salmonis* was identified in diseased salmonids in western Canada, Norway and Ireland (Brocklebank et al., 1992; Evelyn, 1992; Olsen et al., 1993; Rodger and Drinan, 1993; Palmer et al., 1997). Researchers in British Columbia, Canada, reported that a piscirickettsiosis-like syndrome had been observed as early as in 1970 in pink salmon (*Oncorhynchus gorbuscha*) and observed again, in the 1980s, in net pen-reared coho and chinook salmon (Fryer and Mauel, 1997). The agent was isolated from the CHSE-214 salmon cell line and found to be morphologically similar to the Chilean isolate LF-89 and did react with *P. salmonis*-specific polyclonal serum in a fluorescent antibody test.

The *P. salmonis* type strain, LF-89 (ATCC VR 1361), is the best characterized of this group of fish pathogens. It is a non-motile, gram negative, obligate intracellular bacterium, pleomorphic but predominately coccoid, and 0.5–1.5 μm in diameter and occasionally occurs as rings or pairs of curved rods within membrane-bound vacuoles in cultured fish cells and in cells throughout an infected fish. Ultrastructurally, it displays typical gram negative prokaryote membrane and cell wall characteristics. In the 2nd Edition of the Bergey's Manual of Systematic Bacteriology (Boone and Castenholz, 2001), *P. salmonis* has been placed in the Class, Gammaproteobacteria; Order, Thiotrichales and Family, Piscirickettsiaceae based on sequence analysis of the 16S rDNA gene (Fryer et al., 1992; Mauel et al., 1999). This positions *P. salmonis* as more closely related to *Francisella*, *Legionella* and *Coxiella*, that are members of the class Gammaproteobacteria, than to the Genera *Rickettsia* or *Ehrlichia* that are members of the class Alphaproteobacteria.

P. salmonis can be cultured in susceptible fish cell lines, such as CHSE 214, EPC, and CHH, where it produces a cytopathic effect (CPE), rounding of cells, and titers of 10^6 – 10^7 TCID₅₀/ml, but does not replicate in any known cell-free medium. Replication of the type strain is optimal at 15–18 °C, retarded above 20 °C and below 10 °C and does not occur above 25 °C. The type strain LF-89 is sensitive to a broad range of antibiotics (e.g. erythromycin, oxolinic acid, tetracycline) but not to penicillin. *P. salmonis* retains infectivity when suspended in seawater for an extended period (14 days), but rapidly loses infectivity when suspended in fresh water (Lannan and Fryer, 1994). Due to the length of survival time in saltwater, horizontal transmission may occur without a vector. Supporting this concept and indicating the presence of a *P. salmonis*-like bacterium, an

amplicon, with a sequence that was 99.5% similar to the *P. salmonis* 16S rDNA sequence, was produced from Oregon coastal water bacterioplankton DNA with a PCR using *P. salmonis*-specific primers (Mauel and Fryer, 2001).

2. Clinical signs and histologic lesions

Clinical signs and mortality start to occur 6–12 weeks after the smolts are placed in saltwater net pens. Infected fish aggregate in the corners of the net pens near the surface are anorexic. Salmonids infected with *P. salmonis* often show skin lesions that range from slightly raised areas less than 0.5 cm in diameter to hemorrhagic ulcers 2 cm in diameter (Branson and Nieto Diaz-Munoz, 1991). Many of the infected fish are dark and lethargic with some ascities, while others appear externally completely normal. The fish organs are pale (anemia), and there is mild splenomeglia. The kidneys and liver are enlarged, gray with multiple pale foci. Petechial hemorrhages are found throughout the viscera and swim bladder. Hematocrits fall to 2–20% compared to the normal 40–45%. Cream-colored crater-form lesions are present on the liver of chronically infected salmon (Cvitanich et al., 1991; Schäfer et al., 1990; Garcès et al., 1991).

Histologically, gills show multifocal epithelial hyperplasia with mild to moderate necrosis in the hyperplastic tissue. Bacteria are present in areas of severe consolidation of secondary gill lamellae. Gut tissue is severely damaged, with necrosis and sloughing of the epithelial cells. Skin lesions show necrosis of the dermis and epidermis, and some degeneration of sub-dermal musculature. Kidneys are invaded by inflammatory cells and there is generalized necrosis of hemopoietic tissue, with edema and fibrosis. Necrotic thrombi are present in some smaller blood vessels, often with necrotic changes in the vessel endothelium. Enlarged macrophages containing *P. salmonis* bacteria and tissue debris are common. The hemopoietic cells of the spleen display the same pathology as cells of the kidney. Multifocal to general necrosis, inflammation, and increased eosinophilic ground substance are present in the liver. *P. salmonis* cells are present within macrophages, hepatocytes, and found free in the blood.

3. Diagnosis

Isolation of the bacterium in susceptible fish cell lines is the most sensitive method for detecting *P. salmonis* (Fryer and Lannan, 1996). Unfortunately, bacterial isolation is often complicated by field contamination due to the difficulty of aseptic collection of specimens in the field. Normally, contamination can be overcome with the use of low levels of antibiotics but, in this case, *P. salmonis* would also be susceptible thus rendering antibiotics contraindicated. Therefore, the initial diagnosis of piscirickettsiosis is normally made by examining Gram, Giemsa, methylene blue, or acridine orange-stained kidney imprints or smears. Serologic methods, e.g., immuno-fluorescence (Lannan et al., 1991; Lannan and Fryer, 1991) or immunohistochemistry (Alday-Sanz et al., 1994) are used to confirm. A *P. salmonis*-specific nested polymerase chain reaction (PCR) has been developed that can also be used for confirmation or assaying brood stock. The nested PCR assay allows

detection of less than one *P. salmonis* tissue culture infectious dose 50 (TCID₅₀) (Mauel et al., 1996). Marshall et al. (1998) developed a different PCR assay using the 16S–23S rDNA ITS region of the ribosomal operon. This assay was able to detect 10–100 *P. salmonis* cells per 20 µl blood serum. PCR assays require specialized equipment and may be cost prohibitive for routine health checks, but the PCR specificity and sensitivity make it a valuable research and confirmation tool.

In the 1990s, bacteria similar to those implicated in the salmon epizootics in Chile and western Canada caused low level mortalities in Atlantic salmon held in sea water cages in Norway (Olsen et al., 1993, 1997), and the east coast of Canada (Cusack et al., 1997; Jones et al., 1998). All of these agents have since been identified as *P. salmonis* using a polyclonal fluorescent antibody detection test developed by Lannan et al. (1991). Although piscirickettsiosis occurs in salmonids in numerous locations worldwide, the virulence of the disease varies greatly with location. Losses (>90%) occur in coho salmon held in seawater in Chile where extensive epizootics consistently recur. In other areas, such as Norway, Ireland, and Canada, the mortality is as low as 0.06% and the outbreaks sporadic. Controlled experiments performed in an aquatic biologic level three containment facility concluded that the Chilean isolate, LF-89, was significantly more virulent than the isolates from Norway and British Columbia (House et al., 1999).

Little is known of the source of these infections or the reservoir of the etiologic agent, although it seems likely that they may originate from one or more naturally occurring aquatic host. The normal mode(s) of transmission of *P. salmonis*, whether horizontal, vertical, or via a vector, is also unknown. Studies indicate that horizontal transmission can occur (Cvitanich et al., 1990; Almendras et al., 1997). A study by Smith et al. (1999) suggested that the skin and gills are routes of entry of *P. salmonis*. *P. salmonis* has been found in upstream migrating coho salmon and these fish may have a possible role in the ecology and spread of the disease (Pérez et al., 1998). No vector for *P. salmonis* has been identified and even though *P. salmonis* has been observed in gonads (Larenas et al., 1996), the low numbers of infections reported in fresh water indicate that vertical transmission must be rare.

4. Piscirickettsiosis-like syndromes

Grant et al. (1996) reported the isolation of a PLO from an epizootic in farmed salmon in Scotland that was associated with neurological signs and mortality. When this isolate was injected into disease-free fish and held at 12 °C, mortality reached 100% within 18 days. This isolate did not react with anti-*P. salmonis* antibodies in a latex agglutination test indicating that this isolate is an antigenic variant of *P. salmonis* or a different bacterial species.

A PLO was reported by Khoo et al. (1995) in moribund specimens of the blue-eyed plecostomus (*Panaque suttoni*), a fresh water tropical fish, shipped to the United States from Colombia. Small, discrete, coccoid organisms were observed in the cytoplasm of monocytes and in tissue macrophages of the kidney, spleen, heart and occasionally the liver. The PLOs stained similar to *P. salmonis*; gram negative, Macchiavello's negative, blue with Giemsa and H&E. Granulomatous lesions in the spleen, kidney and heart were

similar to piscirickettsiosis in salmonids but there was no vasculitis or thrombosis. Mortalities of 100% are common in the affected shipments (R. Yanong, University of Florida, personal communication).

Camps et al. (1996) found PLOs in the brain of moribund farmed juvenile seabass (*Dicentrarchus labrax*) from the Mediterranean coast, southern France. The fish exhibited abnormal swimming and whirling behavior and nervous tissue necrosis was observed in the brain. High mortality (20%) was associated with this PLO infection. Also, in the Mediterranean, Athanassopoulou et al. (1999) recently reported PLO infections in cultured seabass (*D. labrax*) in Greece. Twenty days after transfer to sea pens the fish exhibited erratic swimming and lethargy, with mortality to 80% in the colder months. The morphology of the bacteria was similar to *P. salmonis*, but the pathology of the infection was localized to the meninges of the brain, retina of the eyes, the adjacent subcutaneous and integumental tissue and the olfactory nerve.

Chen et al. (1999) reports the isolation in cell culture of a PLO associated with high mortality from cultured white seabass (*Atractoscion nobilis*) in southern California. Infected fish demonstrated enlarged kidney and spleen with some fish showing focal pustules or discoloration in the liver. This isolate did not react with anti-*P. salmonis* polyclonal antibodies in an indirect fluorescent antibody test (IFAT) but *P. salmonis* cells did react strongly to anti-white seabass PLO rabbit serum in an IFAT. When the white seabass PLO was injected interperitoneal into juvenile salmon it was found to be highly virulent inducing 80% mortality within 10 days.

An epizootic disease affecting six species of tilapia in both marine and fresh water ponds was reported in 1992 in Taiwan (Chern and Chao, 1994; Chen et al., 1994). The disease originated at a single farm in southern Taiwan and spread to approximately 37 facilities around the island. Mortality was greater than 75% in severe outbreaks and averaged around 30%. The etiologic agent was described by Chern and Chao (1994), as gram negative, able to grow on tilapia ovary (TO₂) and epithelioma papulosum cyprini (EPC) cell lines, but not able to grow on nine different artificial media and likely is a member of the Rickettsiaceae. Koch's postulates were fulfilled using tissue culture grown bacteria. Chern and Chao (1994) also challenged eight pet trade cichlids (*Cichlasoma managuense*, *C. citrinellum*, *C. severum* (Albina), *Pterophyllum altum*, *P. scalare*, *P. scalare* var., and *Cytotilapia frontosa*, with the PLO by intramuscular injection (IM) or cohabitation. No disease signs were evident in the IM-injected cichlids and only *C. managuense* developed PLO disease signs by cohabitation.

Clinically, diseased Taiwanese tilapia, were dark in color, emaciated and demonstrated abnormal swimming behavior. Frequently, the fish exhibited skin lesions and exophthalmia. The spleen was generally enlarged from 5 to 50-fold. White nodules were often present scattered throughout most of the organs. Experimental infections produced higher mortalities at 15 °C than at 30 °C. In cell culture, the bacteria caused a CPE of rounding and clustering of cells and developed titers of 10⁶–10⁷ TCID₅₀/ml. The bacteria were found within cytoplasmic vacuoles in morula-like masses and measured approximately 0.86 × 0.24 µm. Horizontal transmission of the disease was demonstrated by cohabitation of infected fish and uninfected fish (Chern and Chao, 1994; Chen et al., 1994).

Similarly, in the mid 1990s a PLO in tilapia (*Oreochromis mossambicus* and *Sarotherodon melanotheron*) causing severe mortalities was detected in the island of Oahu, Hawaii

(J. Brock, Aquaculture Development Program, State of Hawaii, personal communication). Cultured and wild tilapia stocks were affected. Losses exceeding 60% occurred resulting in a large drop in income for tilapia farmers. As a result control measures were instituted to prevent the transfer of tilapia to other Hawaiian islands. Since that time the severity of the disease has lessened but the disease is still present and the transfer of fish is still restricted.

The PLO-infected Hawaiian tilapia are dark, with external skin lesions and exhibited abnormal swimming behavior. The gills showed epithelial hyperplasia, with mild to severe consolidation of secondary lamellae. Multiple granulomas were observed in gills, spleen, kidney and the choroids gland. The crater-form liver lesions diagnostic of *P. salmonis* in salmon were not observed. No amplification product was observed from DNA extracted from Hawaiian tilapia PLO-infected tissues with a *P. salmonis*-specific PCR protocol developed by Mauel et al. (1996). No fluorescence was observed when spleens from PLO-infected tilapia were tested by the *P. salmonis*-specific fluorescent antibody test developed by Lannan et al. (1991). These results suggest that the Hawaiian tilapia PLO has different surface antigens than *P. salmonis* and may be a new species or a *P. salmonis* antigenic variant.

During 2000 and 2001, we examined tissues sent to our laboratory from tilapia aquaculture operations in Jamaica, Indonesia, southern California and Florida, affected by epizootics having similar characteristics to piscirickettsiosis. Histologically, splenic granuloma lesions and gram negative bacteria similar to those observed in previous PLO epizootics were observed.

Chen et al. (2000) recently reported a PLO in pond-cultured grouper (*Epinephelus melanostigma*) from Taiwan. The clinical signs and histological lesions were similar to *P. salmonis* in salmonids and the PLO in tilapia. The bacteria occur in cytoplasmic vacuoles in CHSE-214 cells, the size and morphology of the bacteria were similar to *P. salmonis*, but did not produce CPE. The bacteria were observed in macrophages in the gills and spleen stained with *P. salmonis*-specific immunohistochemistry.

5. Treatment and prevention

Branson and Nieto Diaz-Munoz (1991) note that fish, positive for *P. salmonis* but not demonstrating any clinical signs of disease or experiencing any mortalities, have been found in net pens. They suggest that stress due to a variety of factors, such as smolt transfers, water temperature changes and severe storms, play a role in the development of piscirickettsiosis. Thus, reducing stress (i.e. by reducing fish density, the number of net changes and by reducing the number of size gradings) has shown value in reducing epizootics by *P. salmonis*. Additionally, Cassigoli (1994) suggests the following control measures: immediate removal of dead and moribund fish from the net pens, reducing the number of fish per site and leaving an area fallow for a period of time.

P. salmonis has been found to be sensitive in vitro to streptomycin, gentamicin, tetracycline, chloramphenicol, erythromycin, oxytetracycline, flumequine, imequil, oxolinic acid, sarafloxacin, clarithromycin and resistant to penicillin, lincomycin, furazolidone, and sulfonamide-trimethoprim (Fryer et al., 1990; Cvitanich et al., 1991; Gaggero, 1995). The effectiveness of oral antibiotic treatment in the field has not been consistent and,

overall, mortalities in salmonid facilities continued to increase through the 1990s (Cassigoli, 1994). The failure of antibiotics to control piscirickettsiosis could be due to variability of antibiotic dosage per fish when administered orally, the failure of the antibiotic to reach effective levels in the intracellular location of the bacteria, development of bacterial resistance to the antibiotics and/or saltwater cation inhibition of the antibiotics (Barnes et al., 1995).

Control of PLO has been only slightly more encouraging. In IM and oral chemotherapy trials, Chern and Chao (1994) demonstrated that the PLO was sensitive to oxytetracycline, tetracycline, chloramphenicol and erythromycin (in decreasing order of sensitivity), but resistant to penicillin G. Chern and Chao (1994) found that an orally administered dose of 30–50 mg/kg of fish body weight of oxytetracycline decreased mortality from the PLO. Although this protocol remains to be tested on a broader scale, it is somewhat encouraging but still not optimal.

The inconsistent outcome of treatment with antimicrobials has encouraged research into the development of vaccines. A 1:2 diluted lysed cell culture supernatant of formalin killed *P. salmonis* bacterin was found to have a protective effect in a trial using coho salmon (*O. kisutch*). Contrastingly, a 10× centrifugation concentrated preparation of the bacterial antigen actually increased mortality; however, this trial was not considered stringent because the cumulative mortality was low in the control and treatment groups and an additional pathogen, *Renibacterium salmoninarum*, was infecting the salmonids (Smith et al., 1995). Although further research is needed, the protective effect of the 1:2 diluted lysed cell culture supernatant is encouraging for the development of a vaccine.

Several reports have identified *P. salmonis* antigens that have potential for use in a recombinant vaccine (Barnes et al., 1998; Kuzyk et al., 1996; Jamett et al., 2001). Using polyclonal rabbit sera, Kuzyk et al. (1996) identified four *P. salmonis* protein antigens, with relative molecular weights of 51, 54, 60, and 65 kDa, and two carbohydrate antigens with relative molecular weights of 16 and 11 kDa. The salmonid immune humoral system does not appear to react strongly with *P. salmonis* antigens. However, several *P. salmonis* protein antigens between 10 and 70 kDa in size and a carbohydrate antigen with a relative size of approximately 11 kDa were identified by Kuzyk et al. (1996) from reactions against convalescent sera from coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*). The rabbit and salmonid immune systems appear to react to different antigens.

Kuzyk et al. (2001a) identified immunoreactive clones using a rabbit polyclonal. One of the clones encoded a 17 kDa outer surface protein (OspA) with 62% amino acid sequence homology to the 17 kDa outer membrane lipoprotein of *Rickettsia prowazekii*. This protein has only been found previously in members of the genus *Rickettsia*. It was shown that convalescent coho salmon sera reacted strongly against OspA. When coho salmon that had been vaccinated with OspA produced in *Escherichia coli* were challenged with *P. salmonis*, a survival rate of 59% was achieved, showing a high level of protection. The authors then added T cell epitopes from tetanus toxin and measles fusion protein to the recombinant OspA vaccine. These antigens are universally immunoreactive in mammalian systems and it was hoped that their addition would improve the efficacy of the OspA vaccine. A threefold increase in efficacy was elicited by the OspA combined vaccine (Kuzyk et al., 2001b).

In conclusion, *P. salmonis* was the first RLO recognized as a pathogen of fish. Since its identification a decade ago, numerous microscopic observations and field isolations of PLOs have taken place worldwide in a variety of fish species. Many of these agents have been identified as *P. salmonis* by serologic and DNA sequencing methods, but the relatedness of many others to *P. salmonis* remains unknown. Epizootics caused by these agents often result in high mortalities and great economic losses. For example, the Chilean aquaculture industry has shifted its initial emphasis on the more commercially desirable but highly *P. salmonis*-sensitive coho salmon to the less *P. salmonis*-sensitive Atlantic salmon. Unfortunately, even with the shift to Atlantic salmon some Chilean aquaculture facilities regularly suffer from mortalities over 30% and piscirickettsiosis remains the major aquaculture disease problem (P. Smith, personal communication). In Canada, Ireland and Scotland, reports of piscirickettsiosis have been increasing for the last 5–10 years and farmers are experiencing economic losses. In Europe and California, *Piscirickettsia*-like organisms (PLOs) have been observed in and isolated from seabass species and epizootics have had high mortalities. In Taiwan and Hawaii, PLOs have wreaked havoc on tilapia farms; 37 Taiwanese farms suffered mortalities up to 95%, and in Hawaii, the transport of fish between Oahu and other Hawaiian islands is now restricted with some farmers questioning the feasibility of farming tilapia.

The studies of *Piscirickettsia*-like infections in fish are rapidly evolving. It is apparent that these agents occur throughout the world, in both fresh and saltwaters, have broad host range, may cause high mortality, and are easily transported between premises. *Piscirickettsia*-like bacteria are recognized with increasing frequency from fish around the world in both fresh and saltwaters. The PLOs observed in and/or isolated from non-salmonid fish have not been characterized sufficiently to determine their relationship to *P. salmonis* and requires additional study. An understanding of the antigenic characteristics of the PLOs and the pathology of the disease syndrome is vital to develop prevention and control strategies.

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SHORT COMMUNICATION

Extracellular survival of *Piscirickettsia salmonis*¹

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Piscirickettsia salmonis is a rickettsial pathogen of salmonid fish (Fryer, Lannan, Giovannoni & Wood 1992). It causes the disease piscirickettsiosis, and is the first rickettsial agent to be implicated in the aetiology of a major fish disease.

Piscirickettsiosis has had a substantial economic impact on the salmonid aquaculture industry of southern Chile. It was first observed there in coho salmon, *Oncorhynchus kisutch* (Walbaum), held in salt water (Bravo & Campos 1989), and since that time epizootics have predictably occurred at seawater sites in the spring and autumn of the year, when water temperatures are ca 9–16°C. However, there is only one report of piscirickettsiosis in fresh water (Bravo 1994).

Piscirickettsiosis affects all species of salmonids cultured in salt water in southern Chile. In addition to coho salmon, affected fish include rainbow trout, *Oncorhynchus mykiss* (Walbaum), and both chinook, *Oncorhynchus tshawytscha* (Walbaum), and Atlantic salmon, *Salmo salar* L. A disease, indistinguishable from piscirickettsiosis, occurs in a chronic form among salmonids cultured at seawater sites in western Canada (Evelyn 1992), in Ireland (Rodger & Drinan 1993) and in Norway (Olsen, Speilberg, Melby & Håstein 1993).

The *P. salmonis* type strain, LF-89 (Fryer *et al.* 1992), was isolated from a moribund coho salmon collected at a seawater netpen in southern Chile during an epizootic that produced extensive mortality (Fryer, Lannan, Garcés, Larenas & Smith 1990). The *in vitro* characterization of *P. salmonis* (Fryer *et al.* 1990), and elements of the histopathology associated with piscirickettsiosis (Cvitanich, Garate & Smith 1991) have been described.

Little is known of the source of the aetiologic agent in these infections, and its natural mode of transmission has not been identified. Although limited studies suggest direct transmission of *P. salmonis* is possible under experimental conditions (Cvitanich *et al.* 1991), direct transmission of rickettsiae is rare, and with the exception of *Coxiella burnetii*, an alternate host or vector is required (Weiss & Moulder 1984). Extracellular survival of the rickettsial agent in the aquatic environment is a prime requisite for direct transmission to be an important factor in the epizootiology of piscirickettsiosis. Therefore, the present authors conducted a series of experiments to determine the extracellular survival times for *P. salmonis* under varied conditions of temperature and environment.

In the initial experiments, CHSE-214 cell cultures (Lannan, Winton & Fryer 1984) were inoculated with the *P. salmonis* type strain, LF-89, and the spent culture medium harvested when cytopathic effect was complete. Aliquots of this medium containing both the rickettsia

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and host cell debris were titred, then held at 5, 10, 15 and 20°C, and titred again at 7-day intervals for 28 days.

In the second series of experiments, LF-89 from spent cell culture medium was partially purified (Lannan, Ewing & Fryer 1991) to remove foetal bovine serum and host cell debris. Aliquots of this preparation were pelleted, then resuspended in selected media and incubated at 5, 10 and 15°C. The four suspension media tested were fresh cell culture medium (MEM with 10% foetal bovine serum) and three types of filter-sterilized water. These included high-salinity sea water (3.2%), activated-charcoal-dechlorinated fresh water from the Newport, Oregon, USA, municipal water supply, and an artificial stream water prepared from laboratory-grade, distilled, deionized water and formulated to approximate the water chemistry of a local Oregon stream. Each preparation was titred immediately after resuspension and at 2-day intervals for 14 days.

Survival of LF-89 was affected by both temperature and the choice of suspension medium. In the initial experiments, the observed decrease in infectious titre with time was directly related to incubation temperature (Fig. 1). Infectious LF-89 particles were detectable in spent culture medium after 21 days at 5 and 10°C, but the titre fell below the level of sensitivity of the assay (10^2 TCID₅₀ ml⁻¹) at sample times beyond 14 days at 15°C or 7 days at 20°C. No infectious material was detected in any preparation after 28 days incubation.

In preparations of semi-purified LF-89 in which little host cell debris remained, a similar response to temperature occurred (Fig. 2), but survival was most dramatically affected by the choice of suspension medium. In every case, LF-89 did not survive suspension in fresh water of either type tested, and infectious particles could not be detected immediately after resuspension or at any time thereafter. In contrast, LF-89 survived for an extended period in sea water, and at each temperature after one week incubation the titres of the seawater preparations were higher than the comparable preparations in cell culture medium.

Although *in vivo* studies will be required to establish the normal mode(s) of transmission of *P. salmonis*, the extended survival of the rickettsia in sea water at temperatures permissive for piscirickettsiosis makes direct transmission a possibility in the marine environment. However, the rapid inactivation of the rickettsia in fresh water limits the opportunity for direct transmission under these conditions. This may explain why the disease is rarely observed at freshwater sites,

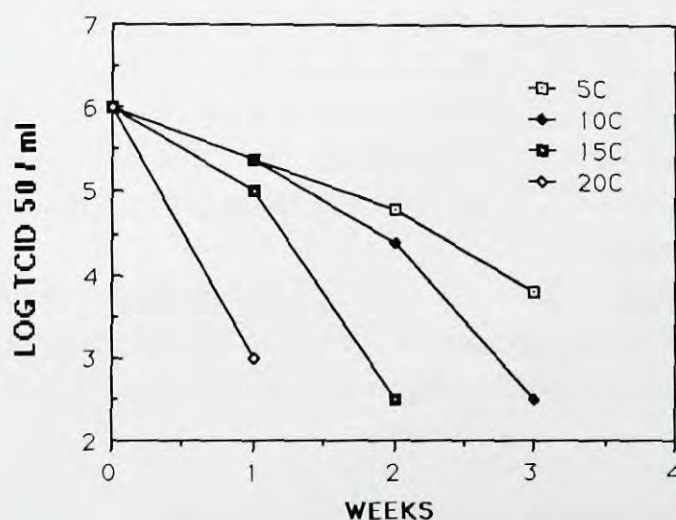


Figure 1. Survival of *Piscirickettsia salmonis*, type strain LF-89, suspended in spent cell culture medium, held at selected temperatures and titred at weekly intervals.

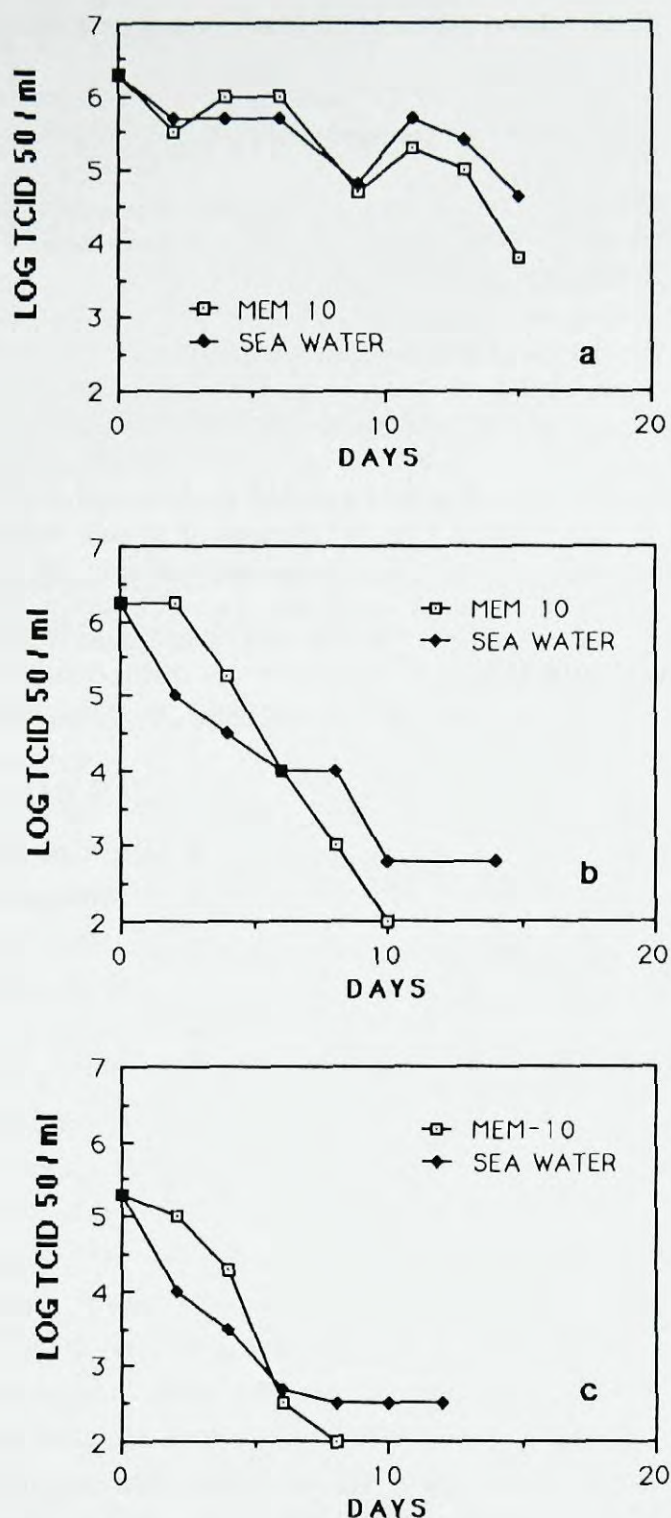


Figure 2. Survival of semi-purified *Piscirickettsia salmonis*, type strain LF-89, resuspended in fresh cell culture medium (MEM-10), high-salinity sea water or two preparations of fresh water, held at (a) 5, (b) 10, or (c) 15 °C, and titred at 2-day intervals for 14 days. In every case, no infectious LF-89 particles were recovered following resuspension in either type of fresh water.

even though it has been demonstrated that piscirickettsiosis develops in fresh water in fish experimentally infected via intraperitoneal injection (Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991).

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Relative virulence of three isolates of *Piscirickettsia salmonis* for coho salmon *Oncorhynchus kisutch*

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ABSTRACT: *Piscirickettsia salmonis* was first recognized as the cause of mortality among pen-reared coho salmon *Oncorhynchus kisutch* in Chile. Since the initial isolation of this intracellular Gram-negative bacterium in 1989, similar organisms have been described from several areas of the world, but the associated outbreaks were not reported to be as serious as those that occurred in Chile. To determine if this was due to differences in virulence among isolates of *P. salmonis*, we conducted an experiment comparing isolates from Chile, British Columbia, Canada, and Norway (LF-89, ATL-4-91 and NOR-92, respectively). For each of the isolates, 3 replicates of 30 coho salmon were injected intraperitoneally with each of 3 concentrations of the bacterium. Negative control fish were injected with MEM-10. Mortalities were collected daily for 41 d post-injection. Piscirickettsiosis was observed in fish injected with each of the 3 isolates, and for each isolate, cumulative mortality was directly related to the concentration of bacterial cells administered. The LF-89 isolate was the most virulent, with losses reaching 97 % in the 3 replicates injected with $10^{5.0}$ TCID₅₀, 91 % in the replicates injected with $10^{4.0}$ TCID₅₀, and 57 % in the fish injected with $10^{3.0}$ TCID₅₀. The ATL-4-91 isolate caused losses of 92 % in the 3 replicates injected with $10^{5.0}$ TCID₅₀, 76 % in the fish injected with $10^{4.0}$ TCID₅₀, and 32 % in those injected with $10^{3.0}$ TCID₅₀. The NOR-92 isolate was the least virulent, causing 41 % mortality in the replicates injected with $10^{4.6}$ TCID₅₀. At 41 d post-injection, 6 % of the fish injected with $10^{3.6}$ TCID₅₀ NOR-92 had died. Mortality was only 2 % in the fish injected with $10^{2.6}$ TCID₅₀ NOR-92, which was the same as the negative control group. Because the group injected with the highest concentration ($10^{4.6}$ TCID₅₀) of NOR-92 was still experiencing mortality at 41 d, it was held for an additional 46 d. At 87 d post-injection, the cumulative mortality in this group had reached 70 %. These differences in virulence among the isolates were statistically significant ($p < 0.0001$), and are important for the management of affected stocks of fish.

KEY WORDS: *Piscirickettsia* · Virulence · Salmon · Disease

INTRODUCTION

In 1989, a novel bacterial fish pathogen, *Piscirickettsia salmonis*, LF-89^T (Fryer et al. 1992), was isolated from netpen-cultured coho salmon *Oncorhynchus kisutch* during an epizootic in Region 10, Chile (Fryer et al. 1990). Losses approaching 90 % were reported (Bravo & Campos 1989), with the onset of mortality occurring 6 to 12 wk after the fish were transferred from freshwater to marine netpens. Moribund fish

were lethargic, dark in color, and swam at the edges of the pens near the surface. Gross pathology included swollen kidneys and enlarged spleens. Other characteristics seen in some, but not all, of the affected fish were anemia (hematocrit 27 % or less), ascites, mottled livers with grey circular lesions, and petechial hemorrhages in the stomach, intestines, pyloric caeca, and visceral fat. Completion of Koch's postulates identified *P. salmonis* as the causative agent of the epizootics, and the disease was named piscirickettsiosis (Cvitanich et al. 1991, Fryer et al. 1992).

Following the initial characterization of the Chilean LF-89 strain of *Piscirickettsia salmonis* (Fryer et al.

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1992), similar Gram-negative intracellular bacterial pathogens of salmonids were described from other areas of the world. In British Columbia, Canada, *P. salmonis* was isolated from netpen-cultured Atlantic (*Salmo salar*) and chinook (*Oncorhynchus tshawytscha*) salmon (Brocklebank et al. 1993). In 1992, *P. salmonis* was recovered from Atlantic salmon cultured in seawater netpens on the west coast of Norway (Olsen et al. 1997). Researchers in Ireland have observed (Rodger & Drinan 1993) and recently isolated from netpen-cultured Atlantic salmon (Palmer et al. 1997) a Gram-negative intracellular pathogen that was identified as *P. salmonis* using an immunohistochemical test with polyclonal antiserum (Alday-Sanz et al. 1994). Additionally, *P. salmonis* was isolated from netpen-cultured Atlantic salmon in Nova Scotia, Canada (Cusack et al. 1997, Jones et al. 1998). None of these isolates was reported to be as virulent as the initial Chilean isolate.

Sequence comparison of the 16S ribosomal RNA genes of *Piscirickettsia salmonis* isolates from Chile, British Columbia, Canada, and Norway (Mauel 1996) demonstrated that these rickettsia-like organisms were closely related. The genetic analysis offered no explanation for the observed differences in losses from piscirickettsiosis in different parts of the world. Factors that could contribute to the reported variation include differences in the virulence of the isolates, host susceptibility, environmental conditions, or co-infections with other pathogens. The purpose of this study was to determine if geographically distinct isolates of *P. salmonis* differed in virulence for coho salmon.

MATERIALS AND METHODS

***Piscirickettsia salmonis* isolates.** Three isolates of *P. salmonis* were used in this study. The type strain, LF-89, was isolated from the kidney of a diseased 2-year-old coho salmon from a seawater netpen site in Southern Chile (Fryer et al. 1990, 1992). The isolate from British Columbia, ATL-4-91, was obtained from netpen-raised Atlantic salmon and was provided by Dr G. Traxler, Department of Fisheries and Oceans, Nanaimo, Canada. An isolate from netpen-raised Atlantic salmon from Norway, NOR-92, was provided by Dr H. P. Melby, National Veterinary Institute, Oslo, Norway. The bacteria were grown at 15°C in CHSE-214 cell cultures (Lannan et al. 1984) using antibiotic-free Eagle's minimum essential medium (Automod, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (MEM-10, Hyclone Laboratories, Inc., Logan, UT) as described by Fryer et al. (1990). Each isolate was passed 5 times in CHSE-214 cells, for a total of 9 passes in cell culture since isolation. Bacte-

rial cells were harvested at 10 to 11 d post-inoculation when the monolayer showed complete cytopathic effect (CPE). The rate of development of CPE appeared similar for all isolates. An endpoint dilution assay (TCID₅₀) was used to determine the infectivity titer of the inocula. This assay used CHSE-214 cells in a 96-well tissue culture plate and was initiated the same day that fish were inoculated. The 96-well plates in the endpoint dilution assay were incubated at 15°C for 28 d, then visually inspected for the presence of CPE. Dilution endpoints were determined by the method of Reed & Muench (1938).

Experimental salmon. Approximately 1000 coho salmon (avg. weight 12 g) were obtained from the Oregon Department of Fish & Wildlife (ODFW), Cascade Locks Hatchery, USA. Previous health examinations did not detect the presence of bacterial or viral fish pathogens. The fish were transported to holding facilities at the Western Fisheries Research Center in Seattle, Washington, USA, and maintained in aquaria provided with 12°C, sand-filtered, UV-treated freshwater from Lake Washington. At the initiation of the experiment, fish were moved into the Biosafety Level 3 (BL-3) containment facility.

Experimental design. Relative virulence of the 3 isolates was determined by comparing the cumulative mortalities and survival times among 3 treatment levels for each isolate. Each treatment and a control group consisted of 3 replicate tanks of 30 fish each. Thus, 90 fish were injected intraperitoneally with 0.1 ml of each of 3 concentrations of each isolate of *Piscirickettsia salmonis* suspended in MEM-10. Fish in the control group received 0.1 ml of MEM-10. Endpoint dilution assays indicated that isolates LF-89 and ATL-4-91 were delivered at doses of 10^{5.0}, 10^{4.0}, or 10^{3.0} TCID₅₀ per fish, and the NOR-92 isolate was delivered at doses of 10^{4.6}, 10^{3.6}, or 10^{2.6} TCID₅₀ per fish.

Maintenance and sampling of fish. Fish were maintained in a series of 27 l tanks in the BL-3 containment laboratory. The tanks were supplied with approximately 2 l min⁻¹ specific-pathogen-free freshwater at an average temperature of 11°C. Effluent from the containment laboratory was treated with 5 mg l⁻¹ chlorine for 30 min, a concentration previously determined to be in excess of that required to kill *Piscirickettsia salmonis* (authors' unpubl. data).

Mortalities were collected daily for 41 d post-injection. Survival curves were estimated using the Kaplan-Meier method, and a logrank test was used to compare the survival curves (Statview, Abacus Concepts, Inc., Berkeley, CA). Mean day to death was calculated for the fish that died during the 41 d period. At 41 d post-injection, the group that was injected with 10^{4.6} TCID₅₀ NOR-92 was still undergoing active mortality, and was maintained and observed for an additional 46 d. The

mean day to death for this group was also calculated for the 87 d period.

Post-mortem examinations were performed on all dead fish. Confirmation of infection with *Piscirickettsia salmonis* included reisolation of the organism from kidney tissue from 50% of the mortalities in each group and microscopic evaluation of stained tissue smears of 100% of the mortalities. *P. salmonis* was reisolated by aseptically removing a portion of the anterior kidney which was homogenized and diluted approximately 1:100 in MEM-10. The homogenate was inoculated into 4 replicate wells of a 96-well plate containing monolayers of CHSE-214 cells in MEM-10 supplemented with 100 U ml⁻¹ penicillin and into 4 replicate wells without penicillin. Cells were incubated at 15°C and examined at 14 and 28 d for the appearance of CPE due to growth of *P. salmonis*. Smears of tissue from the kidney, liver, and spleen were made on 3 replicate slides. The slides were air dried, briefly heated, then fixed in absolute methanol for 5 min and stored at -20°C. Tissue smears from each fish were visually evaluated for the presence of *P. salmonis* by staining using the Giemsa method and confirmed by the indirect fluorescent antibody test (IFAT) described by Lannan et al. (1991).

Ten percent of the mortalities were screened for the presence of *Renibacterium salmoninarum*. Slides were randomly selected and a fluorescent antibody test performed as described by Pascho et al. (1991) using an anti-*R. salmoninarum* polyclonal antibody (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, MD). The kidney samples were visually examined for the presence of the organism using fluorescence microscopy (50 fields of view at 1000×).

RESULTS

Each of the 3 isolates caused mortalities with signs of piscirickettsiosis. Moribund fish were dark, lethargic, and swam near the surface. Generally, the kidneys were swollen and the spleens enlarged, with no discrete lesions visible. Many fish had pale gills indicative of anemia. Some of the fish had ascites. Especially notable was the occurrence of extreme petechial hemorrhaging in the visceral fat and the pyloric caeca. There were no obvious differences in the gross pathology caused by the 3 isolates.

Piscirickettsia salmonis was recovered in cell culture from mortalities in each of the exposed groups. In cases

where bacterial or fungal contamination of the CHSE-214 cell cultures prevented isolation of the organism, *P. salmonis* was identified by IFAT.

The first mortalities began on Day 13 post-injection (PI) in the group injected with LF-89 at a dose of 10^{5.0} TCID₅₀ fish⁻¹ (Fig. 1). The mean day to death in this group was 18 d PI, with peak mortality at 15 d PI, and the cumulative mortality was 97% at 41 d. In the groups exposed to 10-fold lower doses of LF-89, the initial mortalities, mean days to death, and peak mortalities were delayed (Table 1). The cumulative mortalities for the groups receiving doses of 10^{4.0} and 10^{3.0} TCID₅₀ fish⁻¹ were 91 and 57%, respectively. When survival times among groups of fish receiving the high and middle doses were compared using a logrank test, there was a significantly shorter survival time for fish receiving the high dose, and, similarly, the fish receiving the middle dose had shorter survival times than the fish injected with the low dose ($p < 0.0001$ for both comparisons).

Onset of mortality in the ATL-4-91 groups was delayed compared to the groups receiving the LF-89 isolate (Fig. 2). In fish injected with 10^{5.0} TCID₅₀ of the ATL-4-91 isolate, mortality began on Day 15 PI and peaked at 30 d PI, with a mean day to death of 27 d. Cumulative mortality was 92% in this group. A clear dose response pattern, similar to that seen in the groups of fish injected with the LF-89 isolate, occurred in the groups of fish injected with the ATL-4-91 isolate (Table 1). Cumulative mortalities reached 76 and 32% in the groups receiving 10^{4.0} and 10^{3.0} TCID₅₀ fish⁻¹, respectively. The median survival times were 27 and 35 d PI for the high and middle doses, and this differ-

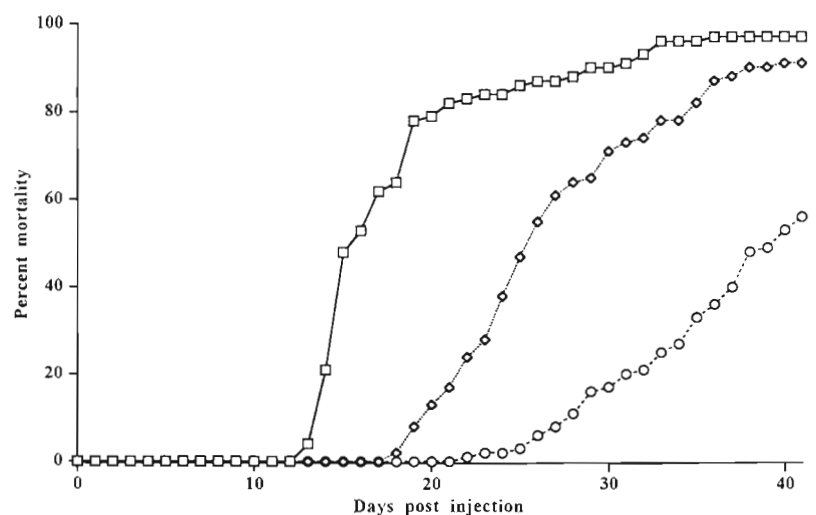


Fig. 1. *Piscirickettsia salmonis* infecting *Oncorhynchus kisutch*. Mean cumulative mortality of coho salmon injected intraperitoneally with different doses of the LF-89 isolate from Chile. Doses used were (—□—) 10^{5.0} TCID₅₀, (---○---) 10^{4.0} TCID₅₀, and (····○····) 10^{3.0} TCID₅₀.

Table 1. *Piscirickettsia salmonis* infecting *Oncorhynchus kisutch*. Mortality among groups of coho salmon held for 41 d following intraperitoneal injection of isolates

Isolate, source	Inoculum (TCID ₅₀)	Initial mortality (d)	Peak mortality (d)	Mean day to death	Median survival time (d)	Cumulative mortality (%)
LF-89, Chile	10 ^{5.0}	13	15	18	16	97
	10 ^{4.0}	18	24	27	26	91
	10 ^{3.0}	22	38	33	40	57
ATL-4-91, British Columbia, Canada	10 ^{5.0}	15	30	27	27	92
	10 ^{4.0}	21	37	32	35	76
	10 ^{3.0}	26	38	36	— ^b	32
NOR-92, Norway	10 ^{4.6}	16	38	33	— ^b	41
	10 ^{3.6}	26	— ^a	33	— ^b	6
	10 ^{2.6}	8	— ^a	33	— ^b	2
Control	0	33	— ^a	34	— ^b	2

^aNot calculated. ^bUnable to calculate from data; less than 50 % died in this group

ence was significant ($p < 0.0001$). No statistical comparison was made with the low dose group because less than 50 % of the fish had died at 41 d PI.

The groups inoculated with the NOR-92 isolate had lower cumulative mortalities and a slower onset and loss rate compared with the groups inoculated with the other isolates (Fig. 3). Fish receiving 10^{4.6} TCID₅₀ of this isolate began dying on Day 16 PI, and the daily number of mortalities increased slowly, with a peak at 38 d PI. At 41 d PI, the cumulative mortality reached 41 %, with a mean day to death of 33 d PI. The observation of this group continued until 87 d PI, when the cumulative mortality had reached 70 %. The mean day to

death at this time was 41 d PI. In the group injected with 10^{3.6} TCID₅₀ fish⁻¹ the cumulative mortality was 6 %, and only 2 fish injected with 10^{2.6} TCID₅₀ NOR-92 died over the 41 d period, for a cumulative mortality of 2 % (Table 1). Death of the first fish in the latter group occurred on 8 d PI and was attributed to bacterial kidney disease (BKD). This fish had a large off-white lesion on the kidney, and examination of the kidney imprint stained with Geimsa, *Renibacterium salmoninarum* FAT and the *Piscirickettsia salmonis* IFAT revealed the presence of *R. salmoninarum*, but not *P. salmonis*. The second fish that died in this group at 33 d PI had piscirickettsiosis. The median survival time was not estimated for any of the groups injected with the NOR-92 isolate, as none of the groups had reached 50 % mortality by 41 d PI.

Two of the 90 control fish died during the experiment (Fig. 3).

When survival data for similar doses of the different isolates were compared, it was found that fish injected with the LF-89 isolate had statistically shorter survival times than fish injected with the ATL-4-91 or NOR-92 isolates ($p < 0.0001$ for all comparisons). Additionally, fish injected with ATL-4-91 had significantly shorter survival times than fish exposed to the NOR-92 isolate ($p < 0.0001$ for all comparisons).

High mortalities in all treatment groups inoculated with isolate LF-89 prevented calculation of LD₅₀ for that isolate. The LD₅₀ values of 10^{3.4} TCID₅₀ fish⁻¹ for ATL-4-91 and 10^{4.6} TCID₅₀

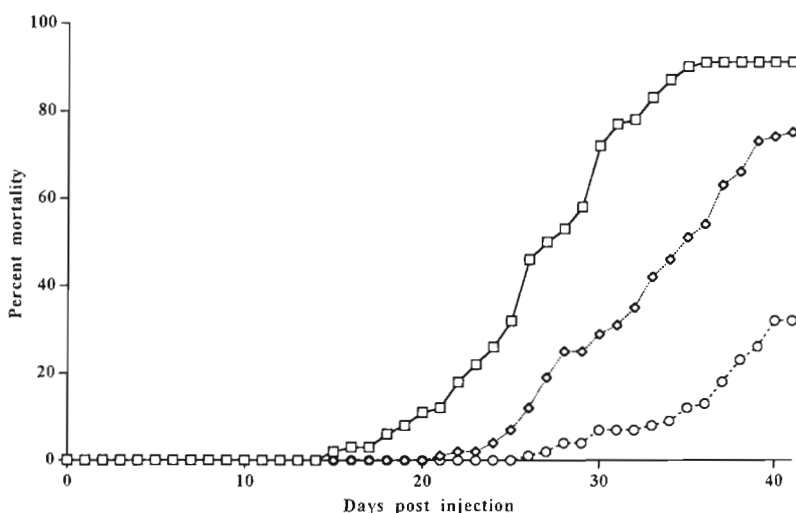


Fig. 2. *Piscirickettsia salmonis* infecting *Oncorhynchus kisutch*. Mean cumulative mortality of coho salmon injected intraperitoneally with different doses of the ATL-4-91 isolate from British Columbia, Canada. Doses used were (—□—) 10^{5.0} TCID₅₀, (---○---) 10^{4.0} TCID₅₀, and (.....○.....) 10^{3.0} TCID₅₀

fish⁻¹ for NOR-92 were calculated based on the mortalities that occurred over the course of the 41 d study.

There were 6 fish out of the 900 (0.7%) in this study in which a lesion due to *Renibacterium salmoninarum* was noted on the kidney, with 1 case occurring in each of the following groups: LF-89 10^{4.0} and 10^{3.0} TCID₅₀, ATL-4-91 10^{4.0} TCID₅₀, NOR-92 10^{3.6} and 10^{2.6} TCID₅₀, and the MEM-10 control group. In these few clinical cases, high numbers of *R. salmoninarum* were present and *Piscirickettsia salmonis* was not detected in kidney tissue impressions from these fish. Analysis of kidney impressions from the mortalities in the study indicated that, in addition to dying of *P. salmonis*, 6% of the fish harbored moderate or low infections with *R. salmoninarum* (40 or fewer bacterial cells in 50 fields observed at 1000×). No one treatment group was more severely affected than another, and because the *P. salmonis* IFAT results were used to confirm the specific cause of death, no fish were removed from the statistical analysis.

DISCUSSION

Results from this study demonstrated statistically significant differences in the relative virulence of *Piscirickettsia salmonis* isolates from Chile (LF-89), British Columbia, (ATL-4-91) and Norway (NOR-92) for coho salmon. Isolate LF-89 caused the most rapid onset of mortality and significantly shorter survival times compared to isolates ATL-4-91 and NOR-92. There was a direct and significant relationship between dose and mortality, with delayed onset and decreased losses in the groups inoculated with lower concentrations of LF-89. The results in this study confirmed and extended challenge studies previously done in Chile with the LF-89 isolate (Garcés et al. 1991). In that study, doses of 10^{5.3}, 10^{4.3} and 10^{3.3} TCID₅₀ were administered to 10 g coho salmon. Mortality in the high dose group began at 16 d post-injection, losses reached 100% by 33 d, and the mean day to death was 18 d. In both studies, the initial mortalities occurred later and the overall losses were less among groups that received the lower concentrations of LF-89. Results from our study and those from Garcés et al. (1991) did not allow the calculation of the LD₅₀ for the LF-89 strain, but Smith et al. (1996) reported that the LD₅₀ for the LF-89 isolate was 10^{2.8} TCID₅₀ ml⁻¹ (10^{1.5} TCID₅₀ fish⁻¹) in 16–17 g coho

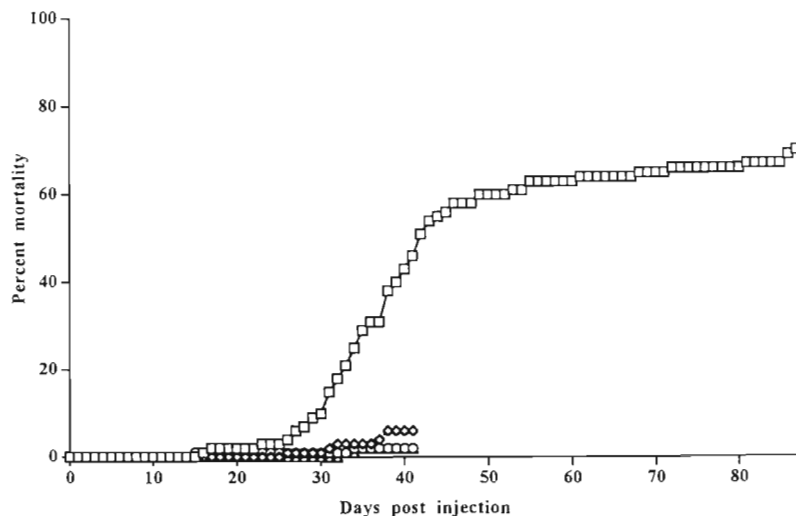


Fig. 3. *Piscirickettsia salmonis* infecting *Oncorhynchus kisutch*. Mean cumulative mortality of coho salmon injected intraperitoneally with different doses of the NOR-92 isolate from Norway and of the negative control group. Doses used were (—□—) 10^{4.6} TCID₅₀, (—○—) 10^{3.6} TCID₅₀, and (—△—) 10^{2.6} TCID₅₀, and (—▲—) Control

salmon. In the present study, 57% mortality had occurred by termination of the 41 d experiment in the group that received a dose of 10^{3.0} TCID₅₀ fish⁻¹, confirming that the LD₅₀ values for the LF-89 strain are slightly below 10^{3.0} TCID₅₀ fish⁻¹.

Time to initial mortality among all groups inoculated with the isolate from British Columbia, ATL-4-91, was delayed compared to groups receiving equivalent doses of LF-89, and the survival time was significantly longer. The highest dose (10^{5.0} TCID₅₀ fish⁻¹) of ATL-4-91 did cause severe losses (92% by 41 d), with a mean day to death of 27 d. As in the LF-89 groups, mortality was directly related to the concentration of bacteria administered. The LD₅₀ of the ATL-4-91 isolate was calculated to be 10^{3.4} TCID₅₀ fish⁻¹. Brocklebank et al. (1993) reported that infection by a rickettsia-like agent (subsequently identified as the ATL-4-91 isolate of *Piscirickettsia salmonis*; G. Traxler, Pacific Biological Station, Nanaimo, British Columbia, pers. comm. 1998) caused a cumulative mortality of approximately 8% among Atlantic salmon and negligible losses among chinook salmon in affected netpens in British Columbia. Homogenates of kidney, liver and spleen from moribund fish injected intraperitoneally into healthy chinook salmon smolts caused mortality starting between 20 and 32 d post-injection, with 50 to 88% of the fish dead by 50 d post-injection (Brocklebank et al. 1993). The number of infectious units injected into the fish was not determined in that study; however, our laboratory studies confirmed that ATL-4-91 was virulent when delivered at a high concentration, although less so than the Chilean isolate, LF-89.

The Norway isolate, NOR-92, was the least virulent of the 3 isolates tested in this study, with only the high dose causing mortality that was significantly greater than that observed in the negative control aquaria. It was not possible to hold all of the groups beyond termination of the experiment; however, we were able to accommodate the high dose group until 87 d PI. Fish in this group had a relatively long onset to death, and at 41 d PI, the cumulative mortality was increasing. By the end of the extended period, cumulative mortality had reached 70%, with a mean day to death of 41 d PI. The lower mortality caused by this isolate in our laboratory study was consistent with the descriptions of piscirickettsiosis epizootics in netpen culture in Norway (Olsen et al. 1997). Although NOR-92 was isolated during a disease outbreak, the bacterium was believed to be directly responsible for only limited mortality, with higher losses incurred when other pathogens, such as infectious pancreatic necrosis virus, were involved (Olsen et al. 1997).

The reasons for the differences in virulence among the isolates are not clear, but our study, using controlled conditions and the same stock of fish, indicates that variation between the isolates is significant. While gross pathology was similar for all of the isolates, histological examination may provide further information. Olsen et al. (1997) reported that the pathology seen in Atlantic salmon infected with *Piscirickettsia salmonis* in Norway was generally comparable to that of infections occurring in salmon from Chile, British Columbia, and Ireland. However, certain signs, such as multifocal gill hyperplasia, dilation of the stomach, and inflammation and necrosis of the gut, that were reported by authors working with affected Chilean coho salmon (Branson & Nieto-Díaz-Muñoz 1991) were not seen in cases from Norway (Olsen et al. 1997) or Ireland (Rodger & Drinan 1993). Additionally, unlike that described in reports from Chile and British Columbia, extensive thrombosis was only observed occasionally among fish dying from piscirickettsiosis in Norway. Explanations for differences in the observed pathology include host factors, such as species and condition, as well as differences in the *P. salmonis* isolates themselves. An investigation of the comparative histopathology over the course of the disease may provide some insight into the mechanisms of virulence.

Previously, alignment of 16S rDNA sequences from the isolates of *Piscirickettsia salmonis* used in this study revealed a very high level (99.4%) of genetic similarity (Mauel 1996, Fryer & Mauel 1997). One isolate from Chile, EM-90, was distinguishable from these isolates in a PCR assay (Mauel et al. 1996), indicating that among certain isolates there are small, but detectable, genetic differences. Using convalescent salmonid antiserum, Kuzyk et al. (1996) observed no

differences between the immunoreactive bands of the LF-89 strain and an isolate from British Columbia (BC-95). Although the isolates tested in this study are very closely related, our study was able to demonstrate significant biological differences among them.

Variation in virulence among isolates of rickettsia is not unprecedented. The existence of strains of *Rickettsia rickettsii* with varying virulence has long been recognized (Parker 1935, Price 1953), and McDade (1990) cites examples of strains of *Rickettsia prowazekii*, *Rickettsia akari* and *R. rickettsii*, the etiological agents of louse-borne typhus, rickettsialpox and Rocky Mountain spotted fever respectively, having differing virulence in experimental animals. Toxins, factors that effect adherence to the host cells, and mechanisms that enhance the ability of a pathogen to invade or survive within the host are known to affect virulence (Finlay & Falkow 1997). However, comparative studies of *R. rickettsii* strains (Anacker et al. 1984, 1986) have not identified specific differences in virulence factors (McDade 1990).

As researchers and aquaculturists become increasingly aware of rickettsia-like organisms in fish, the geographic and species distribution of these bacteria will become more defined. Consideration not only of the presence or absence of *Piscirickettsia salmonis*, but also of characteristics of isolates in an area or stock of fish, may be of value in making management decisions involving both farmed and wild fish. Development of diagnostic techniques capable of distinguishing unique antigens and/or genomic characteristics of the isolates will be important tools that will allow managers to make informed decisions concerning disease outbreaks and the movement of fish.

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Review

***Piscirickettsia salmonis*: a Gram-negative intracellular bacterial pathogen of fish**

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Abstract

Piscirickettsia salmonis is the first Gram-negative, intracellular bacterial pathogen isolated from fish and is a significant cause of mortality in salmonid fish. Recent reports of *P. salmonis* or *P. salmonis*-like organisms from new fish hosts and geographic regions have increased the interest in the bacterium. In this review, the important characteristics of the bacterium including recent taxonomic changes, features of the disease caused by the bacterium including transmission, hosts, reservoirs, diagnostic procedures, and current approaches for prevention and treatment have been discussed. The reader is also directed to other reviews concerning the bacterium and the disease it causes (Fryer & Lannan 1994, 1996; Almendras & Fuentealba 1997; Lannan, Bartholomew & Fryer 1999; House & Fryer 2002; Mauel & Miller 2002).

Keywords: *Piscirickettsia salmonis*, piscirickettsiosis, review, control, diagnosis, epidemiology, taxonomy.

Introduction

A novel disease of fish was first observed in 1989 among coho salmon, *Oncorhynchus kisutch* (Walbaum), reared in marine net pens near Puerto Montt, Chile (Bravo & Campos 1989). The first signs of the disease began 6–12 weeks after fish were transferred from fresh water to sea water and

cumulative mortality ranged from 30 to 90% (Bravo & Campos 1989). Anaemia caused by the systemic spread and replication of an intracellular bacterium was the principal characteristic of the disease (Cvitanich, Garate & Smith 1991), although some fish also showed distinct circular nodules in the liver (Fig. 1A). The bacterium failed to grow on bacteriological media but was isolated using the CHSE-214 cell line (Lannan, Winton & Fryer 1984) by Fryer, Lannan, Garcés, Larenas & Smith (1990). The newly isolated bacterium was shown by subsequent experiments (that fulfilled Koch's postulates) to be the causative agent of the disease in coho salmon (Cvitanich *et al.* 1991; Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991).

The Gram-negative, obligate intracellular bacterium was initially referred to as rickettsia-like (Fryer *et al.* 1990; Cvitanich *et al.* 1991) and was grouped in the family *Rickettsiaceae* and assigned to a new genus and species *Piscirickettsia salmonis* by Fryer, Lannan, Giovannoni & Wood (1992). Subsequently, *P. salmonis* has been moved to a new family, *Piscirickettsiaceae*, that is described in Fryer & Lannan (in press). The disease caused by the bacterium is referred to as piscirickettsiosis, although several other names for the disease appear in the early literature.

Characteristics of the bacterium

Taxonomy

The bacterium isolated from infected coho salmon in Chile was named *Piscirickettsia salmonis* gen.

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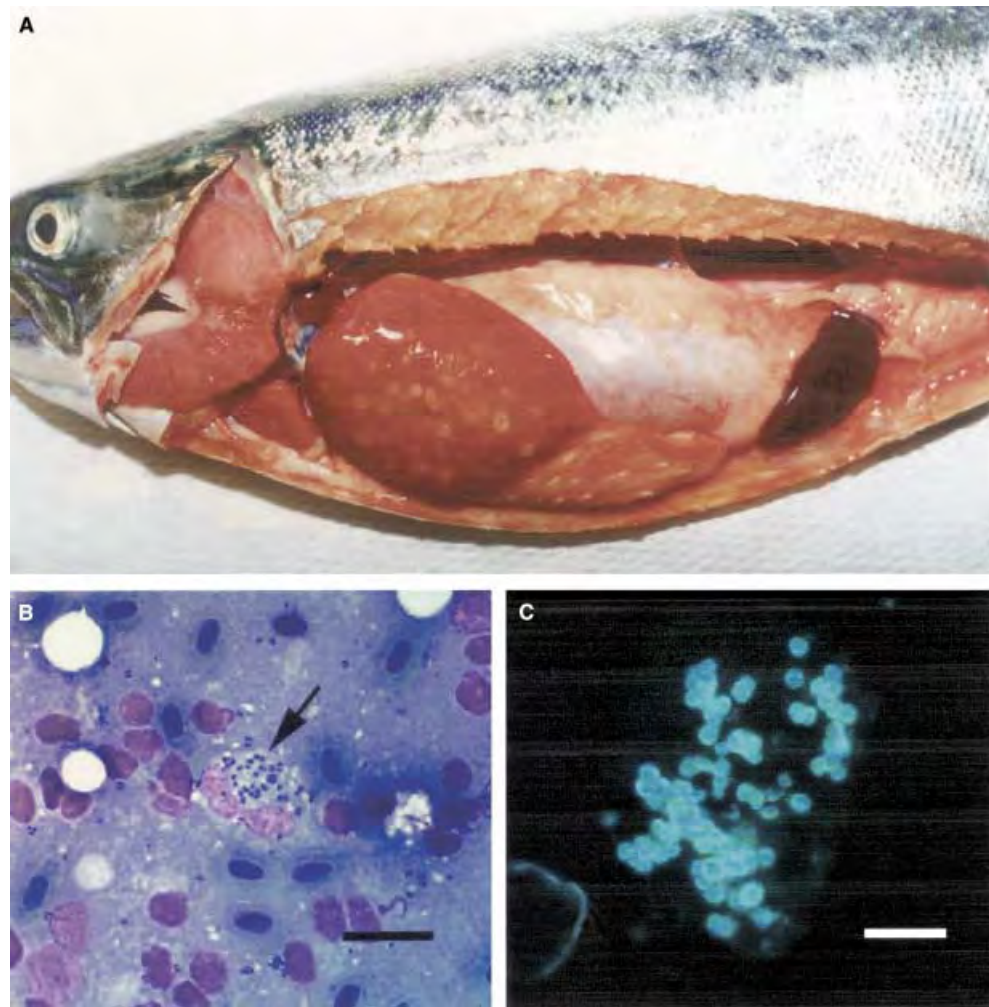


Figure 1 Internal signs of piscirickettsiosis in coho salmon and detection of the bacterium. (A) A juvenile coho salmon with the more chronic form of the disease with circular opaque discolourations evident in the liver. (B & C) Stained smears from the kidney of a coho salmon with piscirickettsiosis: (B) Giemsa stain with arrow showing intracellular bacteria (bar = 20 µm); (C) positive indirect fluorescent antibody test showing individual bacteria (bar = 5 µm).

nov., sp. nov., the type strain was designated LF-89 and the disease named piscirickettsiosis (Fryer *et al.* 1992). *P. salmonis* was never considered a chlamydia because it failed to show the alternating morphological forms associated with this group of bacteria and in addition, it did not react with monoclonal antibodies directed to the genus-specific lipopolysaccharide antigen of chlamydia (Fryer *et al.* 1992). Therefore, at the time, it seemed best to consider *P. salmonis* a member of the family *Rickettsiaceae* with the understanding that major taxonomic changes would occur in the future.

Molecular phylogenetic methods based on sequencing of the 16S rRNA gene have advanced

our knowledge of the taxonomy of *P. salmonis*. Both the nucleotide sequence and the secondary structure of the 16sRNA confirmed the association of *P. salmonis* with the Gammaproteobacteria (Fryer 2002). The Gammaproteobacteria include the genera *Francisella*, *Coxiella* and *Legionella*. On the contrary, the genera *Rickettsia*, *Neorickettsia*, *Cowdria* and *Ehrlichia* are all rickettsia and grouped within the Alphaproteobacteria. The genus *Piscirickettsia* has been placed in a new class within the Gammaproteobacteria and a new family *Piscirickettsiaceae*, clearly separating them from the bacteria of the family *Rickettsiaceae* (Table 1). These recent taxonomic changes for *P. salmonis*

Table 1 Taxonomic outline of *Piscirickettsia salmonis*

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Thiotrichales
Family	<i>Piscirickettsiaceae</i>
Genus	<i>Piscirickettsia</i>
Species	<i>salmonis</i>
Subspecies	–

Source: Garrity & Holt (2001).

[Fryer & Lannan (in press)] are briefly summarized here.

Family Piscirickettsiaceae

The family *Piscirickettsiaceae* contains bacteria that are Gram-negative, aerobic, coccoid, rod or spiral shaped, occasionally pleiomorphic and commonly isolated from the marine environment. All members of this new family belong to the Gammaproteobacteria and are united by similarities in 16S rRNA sequence. The *Piscirickettsiaceae* presently contains five genera, *Piscirickettsia*, *Cycloclasticus*, *Hydrogenovibrio*, *Methylophaga* and *Thiomicrospira*. These genera in the family *Piscirickettsiaceae* are related phylogenetically but have few phenotypic characteristics in common.

Genus Piscirickettsia

The sole species found in the genus *Piscirickettsia* is a Gram-negative coccoid bacterium, approximately 0.5–1.5 µm in diameter, that stains dark blue with Giemsa reagents, and is non-motile, highly fastidious, aerobic, and an intracellular pathogen of fish (Fig. 1B, C). The bacterium replicates within membrane-bound cytoplasmic vacuoles in cells of infected hosts. *Piscirickettsia* can be grown only *in vitro* in fish cell cultures but not on any known artificial medium (Fryer *et al.* 1990). Currently, the genus *Piscirickettsia* contains only one species and this bacterium causes a serious disease among salmonids and other species of fish in marine (Fryer *et al.* 1990) and occasionally freshwater environments (Almendras, Fuentealba, Jones, Markham & Spangler 1997).

Piscirickettsia salmonis has a wide geographic distribution among salmonid hosts in the northwest Pacific and northeast and northwest Atlantic Oceans and the Pacific Ocean in Chile. *P. salmonis* has also been isolated from diseased white sea bass,

Atractoscion nobilis (Ayres), in California, USA (Chen, Yun, Marty, McDowell, House, Appersen, Guenther, Arkush & Hedrick 2000a).

When *P. salmonis* is examined by electron microscopy it displays the typical protoplasmic structure of a prokaryote and the cell wall of a Gram-negative bacterium (Fryer *et al.* 1990; Cvitanich *et al.* 1991; Fryer & Lannan 1996). The cell wall is also the site for attachment of phage particles infecting *P. salmonis* (Yuksel, Thompson, Ellis & Adams 2001). Although it is principally coccoid, pairs of curved rods or rings are also observed. The bacterium replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of susceptible fish hosts (Fig. 1B) or fish cell lines inducing a characteristic cytopathic effect. *In vitro* replication is optimal at 15–18 °C, retarded above 20 and below 10 °C, and does not occur above 25 °C (Fryer *et al.* 1990).

Genetic and antigenic characteristics

Only minor genetic variation has been observed among *P. salmonis* isolates from different salmonid host species or from diverse geographic locations. Sequence data from the 16S rRNA gene are available for five *P. salmonis* isolates representing three salmonid host species and three widely separated geographic regions of North America, South America and Europe (Fryer & Mauel 1997). A comparison of 1527 bp of the 16S rDNA from each isolate demonstrates the formation of a tight monophyletic cluster. Four of the isolates show sequence similarities of >99.4%, while the fifth is also closely related with similarities ranging from 98.5 to 98.9%, but these minor differences were not correlated with either host species or geographic location (Mauel, Giovannoni & Fryer 1999).

Antigens of *P. salmonis* of potential importance in the development of vaccines have been identified by the use of polyclonal and monoclonal antibodies and by their reaction with convalescent salmon sera (Kuzyk, Thornton & Kay 1996; Barnes, Landolt, Powell & Winton 1998; Jones, Markham, Groman & Cusack 1998; Jamett, Aguayo, Miquel, Muller, Arriagada, Becker, Valenzuela & Burzio 2001). Rabbit anti-serum recognizes five to eight antigens with the principal species at molecular weights between 50–60 and 14–16 kDa (Kuzyk *et al.* 1996; Barnes *et al.* 1998). Studies by Kuzyk, Thornton & Kay (1999) stressed the need to utilize *Mycoplasma*-free cell lines to avoid

misidentification of *P. salmonis* antigens. This is of particular importance in vaccine development. One putative outer surface protein (OspA) of *P. salmonis* with a molecular weight of 17 kDa has been more thoroughly characterized and produced in a bacterial expression system (Kuzyk, Burian, Thornton & Kay 2001a).

The disease

In the late 1980s the aquaculture industry in Chile began experiencing considerable mortality in their net pen-reared salmonid stocks (Bravo & Campos 1989). Coho salmon were the main species involved and accounted for the major portion of the fish losses. Cumulative mortality associated with outbreaks frequently ranged from 20 to 30%, reaching 90% in certain cases (Bravo & Campos 1989).

A variety of anti-microbial agents were used in an effort to control the organism and the disease. None of these compounds provided sufficient control of the agent to warrant their use. In Chile, oxolinic acid is frequently the drug of choice but provides less than the desired control of the disease (P. Smith, personal communication).

The external and internal signs of the disease in coho salmon are well described by Cvitanich *et al.* (1991). The disease begins after fish are introduced into the seawater net pens. This has led to the assumption that the bacterium is of marine origin, presumably from a marine fish host. The disease, while still a major problem to the Chilean salmon industry, is reported to be less severe today than in earlier years. This is presumed to be due to a shift from rearing coho salmon to the more resistant Atlantic salmon, *Salmo salar* L., and improved methods of prevention and control procedures once outbreaks begin (P. Smith, personal communication).

Gross pathology

External

A range of signs of infection may be present in salmonids infected with *P. salmonis*. Severely affected fish are dark in colour, show inappetence, are lethargic and swim near the surface or edges of the cages (Branson & Diaz-Munoz 1991; Cvitanich *et al.* 1991; Brocklebank, Speare, Armstrong & Evelyn 1992; Olsen, Melby, Speilberg, Evensen & Hastein 1997; Evelyn, Kent, Poppe & Bustos

1998). On the contrary, less affected fish may show no abnormal external signs. An erratic swimming behaviour has been reported in certain cases in coho salmon and the bacterium was isolated from the brain of affected fish (Skarmeta, Henriquez, Zahr, Orrego & Marshall 2000). Skin lesions, appearing as small white patches that can progress to shallow ulcers, may also be present on some fish. Perhaps the most consistent external signs observed during *P. salmonis* infections are pale gills resulting from a significant anaemia. Haematocrits may decline to values as low as 2%, with an average of 18.5% in infected fish compared with the normal range of 35 to 50% (Branson & Diaz-Munoz 1991; Cvitanich *et al.* 1991).

Internal

The systemic nature of the disease is evident, as several organs and tissues become involved (Fig. 1A). The most common internal signs are a swollen and discoloured kidney and an enlarged spleen. In addition, ascites may be present and haemorrhages on the visceral fat, stomach, swim bladder and body musculature can also occur (Schäfer, Alvarado, Enriquez & Monrás 1990; Cvitanich *et al.* 1991). The livers of more severely affected fish (<20%) are pale and may exhibit cream-coloured circular opaque nodules of 5–6 mm diameter (Branson & Diaz-Munoz 1991; Cvitanich *et al.* 1991; Olsen *et al.* 1997).

Microscopic pathology

The most prominent microscopic lesions are found in the liver, kidney, spleen and intestine but pathological changes in the brain, heart, ovary and gill can also be observed (Cubillos, Farías, Alberdi, Alvarado, Schäfer & Monrás 1990; Schäfer *et al.* 1990; Branson & Diaz-Munoz 1991; Cvitanich *et al.* 1991; Larenas, Hidalgo, Garcés, Fryer & Smith 1995; Palmer, Ruttledge, Callanan & Drinan 1996; Olsen *et al.* 1997; Chen *et al.* 2000a). Multifocal necrosis of hepatocytes in the liver (Fig. 2A) and haematopoietic cells in the parenchyma of the kidney and spleen are features of the more acute phase of the disease. The lamina propria of the intestine is another common site of necrosis, haemorrhage and chronic inflammation (Fig. 2B). Necrosis is followed by granulomatous inflammation that can be observed in the interstitium of the kidney (Fig. 2C) and spleen. This

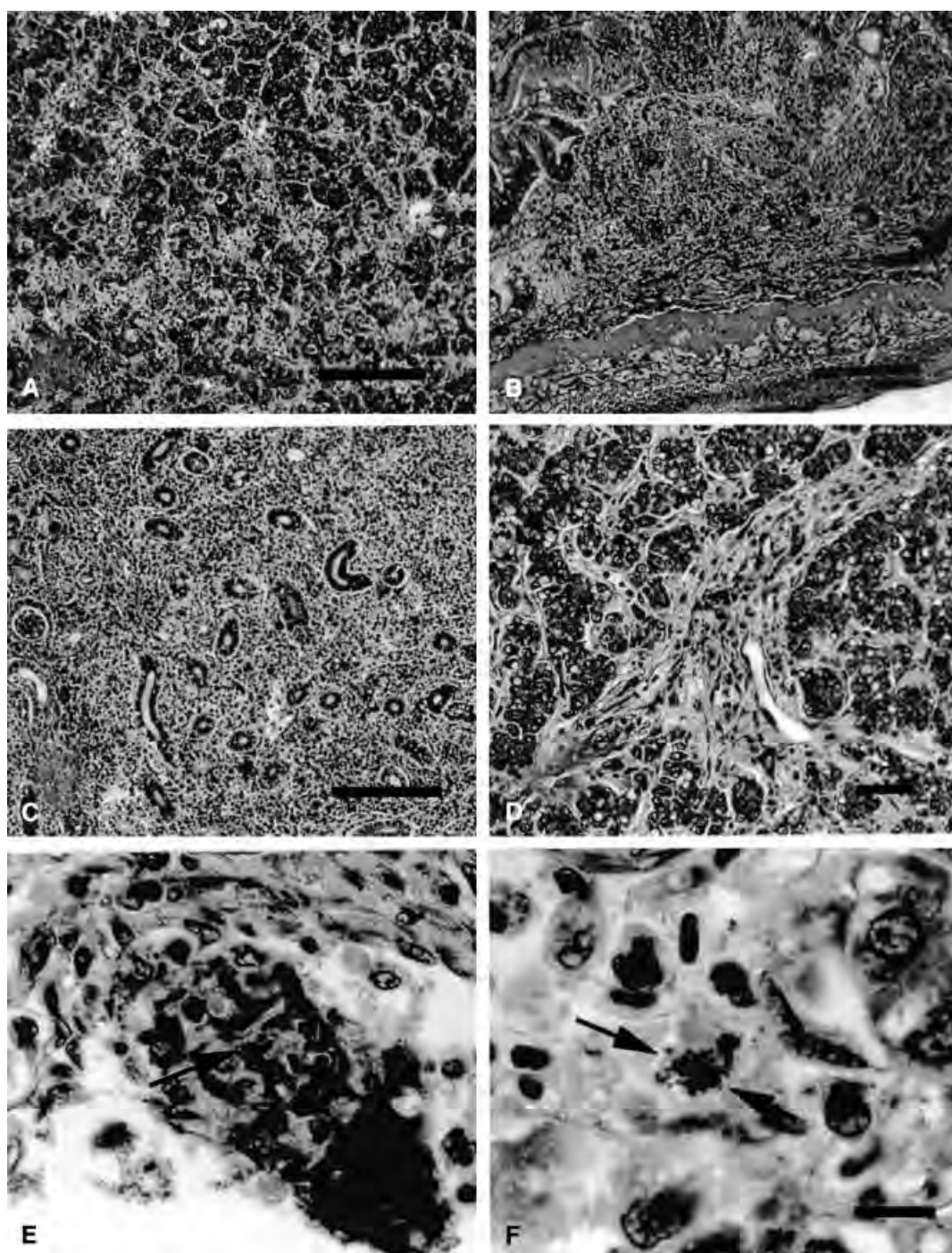


Figure 2 Microscopic lesions associated with *Piscirickettsia salmonis* infection of coho salmon. (A) Multi-focal necrosis of the parenchyma of the liver (bar = 200 μ m). (B) Necrosis, chronic inflammation and haemorrhage in the lamina propria of the small intestine (bar = 200 μ m). (C) Necrosis and granulomatous inflammation in the interstitium of the kidney (bar = 200 μ m). (D) Vasculitis evident around a large vein in the liver (bar = 60 μ m). (E) A fibrin clot with the bacterium present (arrow) (bar = 20 μ m). (F) An intracellular aggregate of bacterial cells in a macrophage in the interstitium of the kidney (arrows) (bar = 10 μ m).

results in the loss of haematopoietic cells that in turn leads to the observed anaemia characteristic of piscirickettsiosis. Vascular and perivascular necrosis are also evident in the liver (Fig. 2D) and intravascular coagulation resulting in fibrin thrombi within major vessels is a common finding (Fig. 2E). Focal areas of necrosis underlie the pale circular lesions observed in more chronically infected fish. In more acute infections, the coalescence of areas of necrosis results in a more mottled appearance to the organ rather than the discrete nodules. Within areas of necrosis, macrophages can be found harbouring intracellular aggregates of *P. salmonis* (Fig. 2F). Meningitis, endocarditis, peritonitis, pancreatitis and branchitis may also be observed with accompanying vascular changes similar to those in the liver and haematopoietic organs. The ovary was also reported to be involved in certain infections in coho salmon by Cvitanich *et al.* (1991). They also reported that infections in coho salmon, rainbow trout, *Oncorhynchus mykiss* (Walbaum), and Atlantic salmon had features in common, but Olsen *et al.* (1997) found no evidence of microscopic lesions in the eye, intestine or gills of infected Atlantic salmon. More recently Skarmeta *et al.* (2000) have isolated *P. salmonis* from the brain of infected coho salmon in Chile. The bacterium recovered from the brain was similar to type strain LF-89 but was not isolated from the kidney and liver of the same fish. The authors proposed that the abnormal swimming behaviour often observed in infected fish may result from previously unrecognized infections of the brain with more highly virulent isolates of the bacterium.

Pathogenesis

Microscopic lesions caused by *P. salmonis* in naturally acquired infections can be found in numerous organs and tissues characteristic of a systemic or septicemic condition. Although the sequential development of the naturally acquired infections has not been reported, initial infections presumably commence when the physical barriers of the skin and/or gills are breached by the bacterium (Almendras *et al.* 1997; Smith, Pizarro, Ojeda, Contreras, Oyanedel & Larenas 1999). Replication of the bacterium results in raised discoloured areas on the skin that may then progress to shallow ulcers, as observed under field conditions and following experimental applications of the bacterium with filter paper patches to the skin (Smith, Contreras,

Larenas, Aguillon, Garcés, Perez & Fryer 1997). Direct dispersion of the bacterial suspension onto the gills also initiates local infections that spread via the blood (haematogenous) and then via major vessels to the parenchyma of numerous organs (Almendras *et al.* 1997). A serosal spread of the bacterium is more characteristic of infections induced by intraperitoneal injections of the bacterium. In injected fish, the capsules of the major organs in the peritoneum are sites of replication prior to invasion of the parenchyma (Almendras *et al.* 1997). In the later stages of infection the internal and microscopic pathological changes observed in fish exposed via different routes become similar, most likely because septicemia eventually occurs even in cases of the serosal spread of the bacterium (Cvitanich *et al.* 1991). The septic nature of infections is demonstrated by the presence of infected macrophages visible in blood smears from heavily infected fish (Cvitanich *et al.* 1991).

Transmission

The transmission of *P. salmonis* in natural infections is horizontal or fish-to-fish and no vector is required. The reservoir for the bacterium is unknown but may include one or more species of fish or other aquatic animals. Almendras *et al.* (1997) demonstrated horizontal transmission of *P. salmonis* under experimental conditions in both fresh and salt water. Experimental work designed to determine if vertical transmission occurs is in progress. The pathogen can occur in the gonad of infected fish and has been observed in the fertilized ova of adult fish inoculated with *P. salmonis* (Larenas, Astorga, Contreras & Smith 1996b). Piscirickettsiosis is less frequently observed in salmonid fish during the freshwater stage of their life cycle than after entry into the marine environment. This suggests that vertical transmission may not be common for this disease agent. Lannan & Fryer's (1994) finding that *P. salmonis* survives longer in salt water than in fresh water perhaps explains why the disease is less frequent in the freshwater environment.

Hosts and geographic range

The principal hosts for *P. salmonis* are salmonid fish (Table 2). Coho salmon was the species involved in the first recorded outbreaks of piscirickettsiosis in Chile (Bravo & Campos 1989). The disease has also

Table 2 Host and geographic range of *Piscirickettsia salmonis*

Host species	Geographic location	Reference
<i>Salmo salar</i>	Canada – Atlantic coast	Cusack <i>et al.</i> (1997)
<i>Oncorhynchus gorbuscha</i> <i>Oncorhynchus tshawytscha</i> <i>Salmo salar</i>	Canada – Pacific coast	Evelyn <i>et al.</i> (1998)
<i>Oncorhynchus kisutch</i> <i>Oncorhynchus mykiss</i> <i>Oncorhynchus tshawytscha</i> <i>Salmo salar</i> <i>Oncorhynchus masou</i>	Chile	Bravo & Campos (1989) Garcés <i>et al.</i> (1991)
<i>Salmo salar</i>	Ireland	Bravo (1994) Rodger & Drinan (1993)
<i>Salmo salar</i>	Norway	Olsen <i>et al.</i> (1997)
<i>Atractoscion nobilis</i>	United States	Chen <i>et al.</i> (2000a)

been reported in rainbow trout, cherry salmon, *Oncorhynchus masou* (Walbaum), and Atlantic salmon in Chile (Garcés *et al.* 1991; Kent 1992; Bravo 1994). Piscirickettsiosis has caused chronic losses among chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and pink salmon, *Oncorhynchus gorbuscha* (Walbaum), in British Columbia, Canada (Evelyn *et al.* 1998). The bacterium has also been found among Atlantic salmon in Ireland (Rodger & Drinan 1993), Scotland (Grant, Brown, Cox, Birkbeck & Griffen 1996) and Norway (Olsen *et al.* 1997). The presence of the agent in Atlantic Canada among farmed Atlantic salmon has been described by Cusack, Groman & Jones (1997). A comparison of the virulence of *P. salmonis* isolates from coho salmon from Chile, British Columbia and Norway demonstrated statistically significant differences (House, Bartholomew, Winton & Fryer 1999). The isolate from Chile (LF-89) was the most virulent inducing losses up to 97% among salmon at the highest doses, compared with 92 and 70% for the isolates from Canada and Norway, respectively.

The only non-salmonid host confirmed to be infected with *P. salmonis* are captive white seabass from California, USA (Chen *et al.* 2000a). Organisms associated with diseases similar to piscirickettsiosis have been detected in additional non-salmonid fish including grouper, *Epinephelus melanostigma* Schultz (Chen, Wang, Tung, Thompson & Adams 2000b), and five species of cultured tilapia (Chern & Chao 1994; Chen, Tung, Chen, Tsai, Wang, Chen, Lin & Adams 1994) in Taiwan. Upon further characterization these isolates may be found to be closely related to *P. salmonis*.

Reservoirs of infection

Marine reservoirs for *P. salmonis* have been suspected since the bacterium was first detected among coho salmon in Chile. This hypothesis was consistent with the onset of infections after transfer to sea water. Also, salmonids are not native to the southern hemisphere and in the absence of strong evidence for egg-associated transmission (Lannan & Fryer 1993) the bacterium must originate from the marine environment. That *P. salmonis* may have been introduced with shipments of eggs to Chile has been proposed but never proven (Bravo & Campos 1989). The bacterium has been found in adult salmon returning to fresh water in Chile (Pérez, Alert, Contreras & Smith 1998) and is found on eggs of experimentally infected Atlantic salmon (Larenas *et al.* 1996b). In addition, the bacterium or a closely related agent has been detected in rainbow trout in fresh water (Bravo 1994; Gaggero, Castro & Sandino 1995; Almen-dras *et al.* 1997). The paucity of outbreaks in fresh water may result from the instability of the bacterium in this environment (Lannan & Fryer 1994).

Despite these observations, vertical transmission has not been convincingly demonstrated as a major route by which the bacterium spreads. The more recent observations of *P. salmonis* (Chen *et al.* 2000a) and *P. salmonis*-like organisms (Chen *et al.* 2000b) among marine fish and in bacterioplankton from sea water (Mauel & Fryer 2001) suggest a potential marine reservoir for the bacterium. Infections of white seabass in southern California (Chen *et al.* 2000a), whose migrations overlap that of Pacific salmon, would be one possible means of

maintaining a marine reservoir for the bacterium. Examinations of non-salmonid marine fish in Chile to date, however, have failed to demonstrate evidence of reservoirs (Garcés, Correal, Larenas, Contreras, Oyanedel, Fryer & Smith 1994). Curiously, evidence for the presence of the bacterium in the parasitic isopod *Ceratothoa gaudichaudii*, but not in *Caligus* sp., was obtained by the indirect immunofluorescent test (IFAT) (Garcés *et al.* 1994). Outbreaks of piscirickettsiosis in salmon may contribute to infections in marine fish reservoirs as fallowing during salmon production cycles has aided in the control of the disease.

Diagnosis and detection

A diagnosis of piscirickettsiosis is based upon presence of the characteristic external and internal signs and microscopic signs of the disease in a salmonid host combined with a demonstration of the presence of *P. salmonis* by one of the several procedures.

Isolation of the bacterium

Piscirickettsia salmonis does not replicate on bacteriological media, but instead must be grown in cell cultures, and therefore escapes detection by routine techniques used for bacterial isolation (Fryer *et al.* 1990; House & Fryer 2002). Additionally, *P. salmonis* is sensitive to many antibiotics used in routine virus isolations *in vitro* (Lannan & Fryer 1991) and will not grow even if inoculated onto suitable host cells if such compounds are included in the culture medium. The tissues of choice for isolation of the agent include kidney, liver and blood during active infection (Lannan & Fryer 1991). No antibiotics should be used in transport or holding media for aseptically collected tissues and tissues should not be frozen but held at 4 °C. Tissues are homogenized in culture media or a balanced salt solution (BSS) and then inoculated on either the CHSE-214 or EPC cell lines (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & de Kinkelin 1983; Lannan *et al.* 1984) at final dilutions of 10^{-2} and 10^{-3} w/v. Cells are incubated at 15–18 °C for 28 days and observed daily for the appearance of cytopathic effects (CPE) that are characterized by plaque-like clusters of rounded cells.

Direct observation of the bacterium in stained preparations

Smears or impressions of the kidney, liver, spleen or infected cell cultures on glass or plastic substrates can be fixed and then stained with Gram, Giemsa, or methylene blue solutions for direct observation of *P. salmonis* within host cells (Fryer *et al.* 1990; Lannan & Fryer 1991). In Giemsa stains, the intracellular bacterium will appear as darkly stained pleiomorphic organisms occurring in coccoid or ring forms often within host cell cytoplasmic vacuoles, frequently in pairs, with a diameter of 0.5–1.5 µm (Fig. 1B).

Confirmation of *P. salmonis*

The fluorescent antibody test can be used to confirm the presence of *P. salmonis* in smears and impressions directly from infected fish tissues, in suspect tissue cultures, and in paraffin-embedded tissue sections (Lannan, Ewing & Fryer 1991; Larenas, Astorga, Contreras, Garcés, Fryer & Smith 1996a; Jamett *et al.* 2001). Appropriately fixed preparations are incubated with rabbit anti-*P. salmonis* serum or mouse monoclonal antibodies and then with fluorescein or other dye-conjugated antibodies directed to rabbit IgG. A similar test can be applied to deparaffinized tissue sections from infected fish with better results obtained if the sections are subjected to microwave irradiation to reveal *P. salmonis* antigens (Larenas *et al.* 1996a). The presence of intracellular bacterial cells with the characteristic size and shape reacting with the specific rabbit or monoclonal antibodies is confirmation of the presence of *P. salmonis* (Fig. 1C). More recently, monoclonal antibodies to *P. salmonis* have been used to detect *P. salmonis* in fish tissues by IFAT and enzyme-linked immunosorbant assays (Jamett *et al.* 2001; Aguayo, Miquel, Aranki, Jamett, Valenzuela & Burzio 2002). The use of monoclonal antibodies may overcome difficulties associated with differences between polyclonal antibody preparations or cross-reactions with other fish or cellular antigens as described by Lannan *et al.* (1991) and Barnes *et al.* (1998). An additional method can be used to confirm the presence of *P. salmonis* in tissue samples or cultures. Polymerase chain reaction (PCR) assays using primer sets designed to amplify target sequences in the small subunit ribosomal (16S) gene (Mauel, Giovannoni

& Fryer 1996) or the internal transcribed spacer (ITS) region between the 23S and the 5.8S ribosomal subunit genes (Marshall, Heath, Henriquez & Orrego 1998) of *P. salmonis* have been developed (OIE 2000). The ITS PCR assay has been modified to provide quantitative values for *P. salmonis* in infected tissues or cells (Heath, Park, Marshall, Prager & Orrego 2000). Detection of a characteristic sized amplicon and sequencing of the product can be used as confirmatory tests for the presence of the bacterium.

Piscirickettsia salmonis might be confused with *Neorickettsia helminthoeca*, the coccoid, Gram-negative, intracellular bacterium that is the aetiologic agent of salmon poisoning disease in canids (Milleman & Knapp 1970). Although *N. helminthoeca* is not a parasite of salmonid fish, salmonids are one of the hosts in the complex life cycle of *Nanophyetus salmincola*, the trematode vector of *N. helminthoeca*. Unlike *P. salmonis*, the geographic distribution of *N. helminthoeca* is restricted to a portion of the Pacific Coast of the USA.

Prevention and treatment

Avoidance

Several husbandry practices appear to aid in minimizing losses caused by *P. salmonis* infection. These include rearing fish at lower densities, allowing farms in a given region to fallow, and avoiding horizontal transmission by holding single year classes of fish at any given site (Evelyn *et al.* 1998). Additionally, if ectoparasites act as vectors, controlling their abundance may be important. The role of vertical transmission remains unclear but diagnostic screening to avoid taking gametes from highly infected adults and subsequent treatment of eggs with iodophors for surface contamination may be of use in avoiding the pathogen.

Immune response and vaccination

The immune response of salmonids to *P. salmonis* infections remains poorly understood, but more recent studies, including successful laboratory vaccine trials, provide optimism for this approach to control of the disease. The humoral response following natural or experimental exposures to *P. salmonis* is weak, although anti-*P. salmonis* antibodies have been detected in the serum of conva-

lescent coho salmon (Kuzyk *et al.* 1996). These antibodies react with protein antigens ranging from 10 to 70 kDa, a carbohydrate antigen of approximately 11 kDa and the OspA lipoprotein (Kuzyk *et al.* 2001a), the latter of which has been further exploited for vaccine development.

Initial attempts to utilize whole cell bacterins to protect salmon from *P. salmonis* infections have shown variable results. In trials with unconcentrated bacterin Smith, Lannan, Garcés, Jarpa, Larenas, Caswell-Reno, Whipple & Fryer (1995) and Smith *et al.* (1997) demonstrated significant protection to natural challenges with the bacterium, while fish receiving adjuvant and concentrated bacterin in the same trial showed no protection or greater susceptibility to infection. In other trials increased survival [35%] relative percent survival (RPS) was observed when coho salmon were injected with whole cell bacterins in oil and water adjuvants (Kuzyk, Burian, Machander, Dolhaine, Cameron, Thornton & Kay 2001b). However, better protection was observed with a vaccine prepared with recombinant OspA alone or fused with T cell epitopes from tetanus toxin and measles fusion protein (Kuzyk *et al.* 2001b). These latter vaccines resulted in RPS values of up to 83%, thus demonstrating the need for field trials aimed at potential control of the disease in seawater netpens.

Chemotherapy

Treatment of active cases of the disease with a number of antimicrobial drugs continues to be utilized as a principal control measure (Branson & Diaz-Munoz 1991; Cvitanich *et al.* 1991; Smith, Contreras, Garcés, Larenas, Oyanedel, Caswell-Reno & Fryer 1996). Orally administered oxolinic acid and oxytetracycline have been reported as useful in the treatment of piscirickettsiosis but with the limitations that the response to these medicated feeds may be slow and require repeated applications (Palmer 1997). The overall efficacy of these treatments is, however, inconsistent (Smith *et al.* 1996). Bacterial replication is inhibited *in vitro* by numerous antibiotics normally used in cell culture or in human and veterinary medicine including gentamicin, streptomycin, erythromycin, rifampin, tetracycline, doxycycline, oxolinic acid and chloramphenicol (Fryer *et al.* 1992). An exception is penicillin that appears to have little effect on *P. salmonis*. The reasons for the inefficacy of antimicrobial therapy for piscirickettsiosis may

reside with the difficulty in obtaining intracellular concentrations of drug sufficient to kill or eliminate the bacterium.

Conclusions

Piscirickettsiosis remains a key problem for the net pen rearing of salmon in South America, North America and northern Europe. Bacteria similar to *P. salmonis* have and probably will continue to be isolated from a number of new marine fish hosts over wide geographic ranges including in more temperate sea water conditions. This suggests that *P. salmonis* and closely related bacteria may have a wide host and geographic range. Although yet to be directly demonstrated, a marine reservoir(s) for the bacterium in salmon rearing areas is likely and will eventually be identified. Treatment of piscirickettsiosis remains problematic, but currently uses prevention procedures and the recent and promising results of vaccination trials provide hope that the disease can be better controlled in the near future.

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Experimental infection and horizontal transmission of *Piscirickettsia salmonis* in freshwater-raised Atlantic salmon, *Salmo salar* L.

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Abstract

Experimental infections with *Piscirickettsia salmonis* via intraperitoneal (IP), oral (PO) and gill (GS) routes were compared, and the importance of physical contact in the horizontal transmission of this organism was investigated. Atlantic salmon, *Salmo salar* L., under-yearling parr raised in fresh water were used in this study. Samples of liver, kidney, spleen, gill and brain were collected weekly for 5 weeks after challenge, and were examined using the indirect fluorescent-antibody technique (IFAT). The pathogen was transmitted horizontally to fish with and without physical contact. However, transmission of *P. salmonis* occurred significantly more rapidly among fish with physical contact. Mortalities occurred in 50% of fish experimentally challenged with *P. salmonis* and their cohabitants. The estimates of the relative risk of dying demonstrated that fish challenged by the IP and GS routes had a significantly higher probability of dying than fish challenged by the PO route ($P < 0.005$). Contact cohabitants with infected fish had a higher probability of death than non-contact cohabitants ($P < 0.005$). The sequential studying using IFAT indicated that a haematogenous pattern of infection occurred among fish infected by oral and gill routes, or by cohabitation. This was different from the capsular (serosal)

infection pattern observed in intraperitoneally inoculated fish. *Piscirickettsia salmonis* was observed within the cytoplasm of leucocytes and renal tubules, the latter indicating that elimination of this pathogen through the urine may be possible. *Aeromonas salmonicida* was also detected (by IFAT) in some of the fish exposed to *P. salmonis*, suggesting that *P. salmonis* may cause immunosuppression, and thus, increase the susceptibility of the host to other pathogens.

Introduction

Salmonid rickettsial septicaemia (SRS), or piscirickettsiosis, is a systemic disease caused by the intracellular pathogen *Piscirickettsia salmonis* (Fryer, Lannan, Garcés, Larenas & Smith 1990), which causes high mortalities and significant economic losses to the salmon industry (Fryer, Lannan, Giovannoni & Wood 1992). This disease was first reported in coho salmon, *Oncorhynchus kisutch* (Walbaum), in Chile (Bravo & Campos 1989), and has since been observed in other salmonid species including rainbow trout, *Oncorhynchus mykiss* (Walbaum), chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and Atlantic salmon, *Salmo salar* L. (Cvitanich, Garate & Smith 1991). Similar rickettsia-like organisms (RLOs) have been reported in Atlantic salmon held in salt water in western Canada (Brocklebank, Evelyn, Speare & Armstrong 1993), Ireland (Rodger & Drinan 1993) and Norway (Olsen, Evensen, Speilberg, Melby & Hastein 1993). Outbreaks in fresh water have been

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recently reported in rainbow trout and coho salmon (Gaggero, Castro & Sandino 1995).

Very little is known about the natural routes of infection, mechanisms of horizontal transmission and pathogenesis of SRS. Hence, it is important to identify the source of infection and to elucidate the mode of transmission of the agent under natural conditions (Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991).

Salmonid rickettsial septicaemia has been experimentally reproduced in coho and Atlantic salmon by intraperitoneal inoculation (Cvitanich *et al.* 1991; Garcés *et al.* 1991). However, the possibility of oral or gill infection by *P. salmonis* has not been studied. Horizontal transmission has occurred in salt water from experimentally inoculated coho salmon to non-inoculated cohabitants (Cvitanich *et al.* 1991), but the possibility of horizontal transmission in fresh water is still controversial (Cvitanich *et al.* 1991; Garcés *et al.* 1991).

Chile is the third largest world producer of Atlantic salmon (FAO 1994), and this production is likely to increase because the importation of eyed eggs of this species has increased by 58% from 1993 to 1994 (Méndez 1995). An understanding of the pathogenesis and transmission of the SRS in Atlantic salmon will be important in developing methods for controlling the disease and ensuring a sustained growth of the industry.

The main objectives of the present project were to compare the experimental transmission of *P. salmonis* via intraperitoneal, oral and gill routes, and to investigate the importance of physical contact in horizontal transmission among Atlantic salmon.

Materials and methods

Culture and identification of the organism

The isolate of *P. salmonis* used in this experiment was obtained from a naturally infected Atlantic salmon with clinical SRS reared in salt water in southern Chile (donated by Dr E. Madrid, Marine Harvest McConnell, Puerto Montt, Chile). The isolate was named strain NCR 1010, and identified as *P. salmonis* by indirect fluorescent-antibody test (IFAT) using a rabbit polyclonal antibody produced against *P. salmonis* strain LF-89 (American Type Culture Collection, VR1361) that was generously donated by Dr J. L. Fryer (Oregon State University, USA).

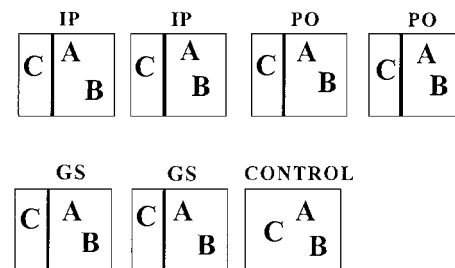


Figure 1 The experimental design. Challenge routes were grouped together. IP, intraperitoneal; PO, oral; GS, gill surface; and control, a non-challenged control group. There were three sources of contact with the pathogen: (A) directly challenged fish; (B) cohabitants in contact with directly challenged groups; and (C) non-contact cohabitants. Each sub-group had 18 fish (there were 54 fish per tank).

Maintenance of the fish

The fish used were under-yearling Atlantic salmon parr, wildstock La Have, from a Department of Fisheries and Oceans Canada-certified disease-free hatchery in Nova Scotia. Three hundred and eight fish weighing 20 ± 2.3 g were acclimatized in fresh water at 11°C, with a flow of 2.0 l min^{-1} in a flow-through system. The water had a dissolved oxygen concentration of 10 p.p.m., and a total hardness of 212 p.p.m. Effluent from tanks used in this study was treated with 5 p.p.m. chlorine for 2 h prior to discharge. All experimental and control fish were exposed to 8 h of artificial light daily, and fed twice a day with an Atlantic salmon #2 granular dry diet (Corey Feed, NB, Canada) at 2% of body weight per day. After one month, the fish were anaesthetized in a solution of benzocaine at a concentration of 50 mg l^{-1} , separated into three groups, and dye-tagged on the caudal (group A), pectoral (group B) or pelvic fins (group C).

Experimental infections

One week after tagging, fish from group A ($n = 108$) were arbitrarily assigned to three sub-groups to be challenged with *P. salmonis*. Thirty-six fish were anaesthetized as described above, and challenged either by the intraperitoneal (IP), oral (PO) or the gill surface (GS) route. Challenged fish were placed separately in six plastic square tanks of ≈ 60 l, using two tanks per challenge route and allocating 18 challenged fish per tank (Fig. 1). The inocula for all routes consisted of $100 \mu\text{l}$ of diluted supernatant from infected chinook salmon embryo (CHSE-

214) cell line (ATCC CRL 1681), which showed a 100% cytopathic effect (CPE). The final infectious dose, estimated by calculating the 50% tissue culture infectious dose endpoint (TCID₅₀) in a 96-well plate seeded with CHSE-214 (Hsiung 1994), was 1.48×10^2 TCID₅₀ ml⁻¹.

Intraperitoneal injections were performed using a 1-ml syringe and a 26-G $\times$ 3/8 inch needle (Becton Dickinson & Co., Rutherford, NJ, USA). A 10-cm-long piece of flexible PVC Tygon tubing with a 0.1 mm internal diameter and a 0.3 mm external diameter (Norton Co., Akron, OH, USA) was connected to the needle to perform oral and gill challenges. For oral inoculations, the fish were gently intubated by inserting the tube $\pm$ 1–2 cm into the oesophagus. Gill infections were performed by dispensing the inoculum over the lamella, between the first and second branchial arch of the left gill.

Cohabitants

In each of the six tanks, groups of 18 non-challenged fish were placed in cohabitation with (group B) or without (group C) direct physical contact with inoculated (group A) fish (Fig. 1). Fish in group C were separated from the other two groups by a 2.5 cm-thick double plastic mesh and occupied one-third of the water volume in order to maintain a stocking density equal to that of the other groups (27 kg m⁻³). The combination of three routes and three modes of contact with the pathogen provided nine experimental groups. Two replicates (tanks) were used for each experimental group. Fifty-six fish were allocated to a separate tank to be used as uninfected controls.

Sampling

Five fish from each of the nine experimental groups and three from the control group were sampled weekly for 5 weeks. During the sampling process, no attempt to select fish showing clinical signs was made. Fish were removed from the tank, sedated in 50 p.p.m. of benzocaine hydrochloride solution and euthanized by spinal severance. The peritoneal cavity was exposed from the left flank for post-mortem examination.

Samples of liver, spleen, head kidney, posterior kidney, brain and the second left branchial arch were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6- μ m thick-

ness. An indirect fluorescent-antibody technique with the rabbit anti-*P. salmonis* polyclonal antibody was used for specific detection of the aetiologic agent in tissue sections, according to the method described by Lannan, Ewing & Fryer (1991). Smears of *Aeromonas salmonicida* subsp. *salmonicida* (Aqua Health Ltd, Charlottetown, PEI, Canada), and tissues of rainbow trout infected with *A. salmonicida* were included to assess the specificity of the reaction. Rabbit anti-*A. salmonicida* (1/400) serum (Aqua Health Ltd) was also used to determine the prevalence of unexpected infection with this organism.

Tissues from fish that died during the study period were collected daily and processed as described above.

Experimental procedures used in this study were performed according to the guidelines of the Canadian Council on Animal Care (Olfert, Cross & McWilliams 1993).

Statistical analysis

The effect of exposure by various routes and type of contact on mortality was assessed in SAS (SAS Institute 1988) using survival analysis. Relative risk of mortality and cumulative probability of survival corrected for lethal sampling was calculated using the Cox proportional hazards model (Lee 1980). Decisions on significance were based on $P \leq 0.05$.

Results

Weekly sampled fish and mortalities

A total of 162 fish (50%) died during the 35 days of the experimental trial: 60 from IP injected challenge and their cohabitants; 57 from GS challenge and their cohabitants; and 45 from the PO challenge and their cohabitants. Therefore, all fish had been sampled from the IP and GS groups for 28 days post-challenge (DPC), whereas the PO and control groups were sampled for 35 DPC. None of the control fish died during the experimental period.

Mortalities were first detected at 12 DPC in the PO challenged fish and the GS challenge contact cohabitants with fish (Table 1). Mortalities corrected for lethal sampling were compared by using life tables for cumulative survival analysis. When comparing routes of challenge, the oral route showed a pattern of mortality that was

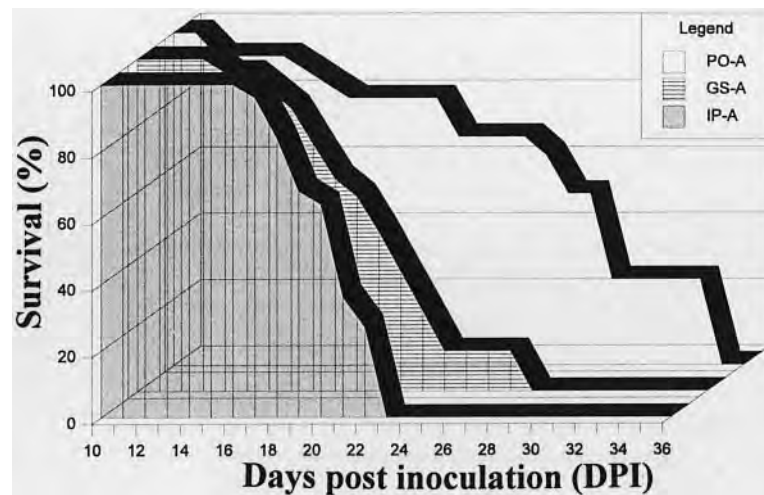


Figure 2 The cumulative probability of the survival of fish with a comparison of challenge routes (directly challenged fish only). Challenges with *Piscirickettsia salmonis* were intraperitoneal (IP-A), gill surface (GS-A) and oral (PO-A).

Table 1 Mortalities observed in each of the experimental groups. The first and last mortality events are expressed as days post-challenge (DPC). The groups are identified according to the exposure to *Piscirickettsia salmonis*, indicating the challenge route used, and contact with the pathogen as direct inoculation, physical contact with infected fish or water cohabitation

Route*	Contact	No.	Mortality event			
			First (DPC)	Last (DPC)	Days with mortalities	Dead/total (%)
IP	Direct	36	17	23	7	57
	Physical	36	17	27	10	59
	Water	36	19	28	11	50
PO	Direct	36	12	35	24	41
	Physical	36	18	30	13	42
	Water	36	25	35	11	41
GS	Direct	36	14	24	11	45
	Physical	36	12	27	16	64
	Water	36	19	27	9	46
Controls	–	54	0	0	0	0
Total	–	378	–	–	–	–

*Challenge route: IP, intraperitoneal; PO, oral; GS, gill surface.

significantly delayed ($P \leq 0.05$) relative to the IP and gill routes (Fig. 2). However, significant differences between replicates of the orally challenged groups were also detected.

Non-contact cohabitants (group C) in IP, GS or PO challenged groups showed a longer ($P < 0.05$) survival time (delayed pattern of mortality) when compared with directly challenged fish (group A) or contact cohabitant fish (group B) (Fig. 3).

The estimates of the relative risk (RR) of dying for each challenge method are shown in Table 2. In the present study, the lowest risk group corresponded to non-contact cohabitants with orally

challenged fish (route PO, group C). The analysis showed that fish challenged IP and by GS had a significantly higher probability of dying than orally challenged fish ($P < 0.05$). Contact cohabitants with infected fish had a higher probability of death than non-contact cohabitants ($P < 0.05$). In addition, there was interaction between route of challenge and type of contact. The risk of death in IP or GS challenged fish (as opposed to orally challenged fish), and contact cohabitants with IP or GS challenged fish was significantly higher than non-contact cohabitants with orally challenged fish (Table 2).

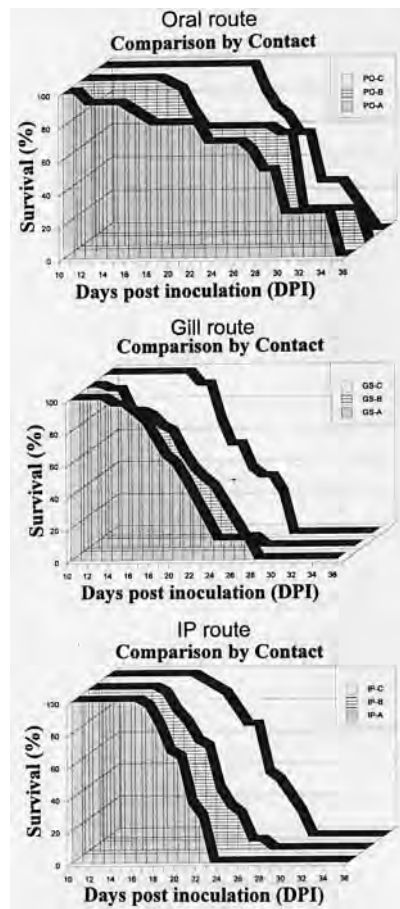


Figure 3 The cumulative probability of the survival of fish with a comparison of contacts in (a) orally (PO) inoculated, (b) gill (GS) challenged and (c) intraperitoneally (IP) inoculated groups with *Piscirickettsia salmonis*: (A) directly challenged fish; (B) contact cohabitants with challenged fish; and (C) non-contact cohabitants with challenged fish.

Table 2 Relative risk (RR) of dying at a given time for each challenge method

Challenge method	Relative risk	P-value
Non-contact cohabitants with orally challenged fish	1.000	–
Contact cohabitants with orally challenged fish	1.090	0.790
Orally challenged fish (PO)	1.090	0.790
Non-contact cohabitants with IP or GS-challenged fish	2.574	0.005*
Contact cohabitants with IP or GS-challenged fish	6.820	0.021*
IP- or GS-challenged fish	6.820	0.021*

*Significantly different from RR = 1.0 ($P \leq 0.05$).

Table 3 Detection of *Piscirickettsia salmonis*, using an indirect fluorescent-antibody technique in tissues of Atlantic salmon sampled at 7, 14, 21 and 28 days post-challenge (DPC)*

Group	Total positive			
	7 DPC	14 DPC	21 DPC	28 DPC
IP-A	4/5	5/5	5/5	N/A
IP-B	2/5	1/5	3/5	1/1
IP-C	0/5	1/5	2/5	0/2
PO-A	2/5	3/5	3/5	5/5
PO-B	0/5	1/5	3/5	4/5
PO-C	2/5	1/5	2/5	5/5
GS-A	1/5	3/5	1/5	0/1
GS-B	0/5	3/5	0/5	N/A
GS-C	2/5	3/5	2/5	3/5
Total (%)	29	47	47	75
Control	0/3	0/3	0/3	0/3

*Route: IP, intraperitoneal; PO, oral; GS, gill surface. Contact: A, direct infection; B, contact cohabitants; C, non-contact cohabitants. N/A, no fish available.

Prevalence of *P. salmonis* in sampled fish

The presence of *P. salmonis* in experimentally infected fish was confirmed using IFAT. Smears of supernatant from infected CHSE cultures were IFAT-positive using the rabbit polyclonal serum (positive control), whereas supernatants from uninfected CHSE cultures (negative control) and smears of *A. salmonicida* were IFAT-negative using this antiserum. Fluorescent rickettsia were observed in sampled fish from all groups exposed to *P. salmonis* (Table 3). By 7 DPC, 29% (13/45) of the samples were IFAT-positive, and this had increased to 47% by 14 DPC and 21 DPC (21/45 and 21/45, respectively), and to 75% (18/24) by 28 DPC.

Patterns of infection with *P. salmonis* in sampled fish

Two distinct patterns of *P. salmonis* infection were observed in directly challenged fish. A capsular (serosal) pattern of *P. salmonis* infection was observed in the liver and spleen in intraperitoneally challenged fish from 7 DPC (Fig. 4). In later samples in this group, *P. salmonis* was also observed to be infecting leucocytes and other parenchymal cells of the spleen, liver and kidney. In contrast, a haematogenous pattern of infection was observed in PO and GS challenged fish, and in contact cohabitants (group B) or non-contact cohabitants (group C). In the latter pattern, the organism invaded the organs from the blood vessels to the surrounding tissues and no

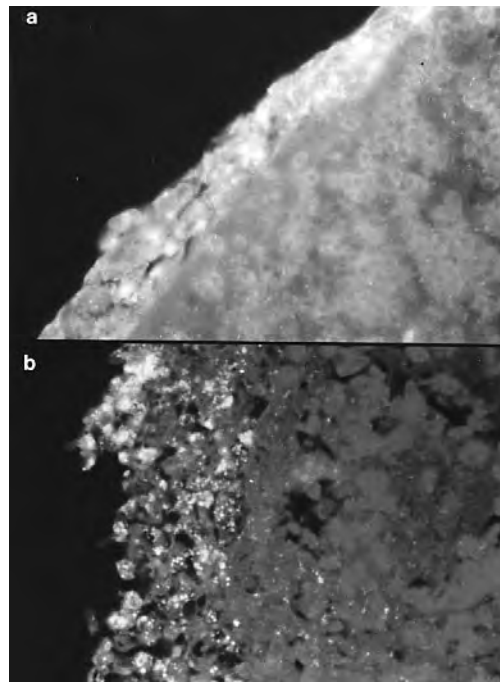


Figure 4 Atlantic salmon spleen: an electron micrograph depicting capsular infection by *Piscirickettsia salmonis* at (a) 7 days and (b) 14 days post-IP inoculation (IFAT, $\times 320$).

evidence of capsular infection by *P. salmonis* was observed in these groups. Leucocytes carrying *P. salmonis* in the cytoplasm were frequently observed within hepatic sinusoids and blood vessels (Fig. 5). In some cohabitant fish (groups B or C), *P. salmonis* was detected as early as 7 DPC (Table 3).

Some fish from each of the nine experimental groups also had bacterial colonies ranging from 3 to 100 μm in diameter, composed of coccobacillary organisms. These bacteria were identified as *A. salmonicida* using IFAT. Analysis by IFAT of tissues from fish experimentally exposed to *P. salmonis* showed that 44% of the sampled fish presented piscirickettsial infection, 27% alone and 17% associated with *A. salmonicida* infection. There were no mortalities among control fish, and none of those sampled (12) had evidence of piscirickettsial infection and only one fish, sampled 35 DPC, had evidence of *A. salmonicida* infection.

IFAT analysis of mortalities

Using the anti-*P. salmonis* serum, foci of fluorescing microorganisms were detected in the liver and spleen serosa of intraperitoneally inoculated fish, and these

occasionally extended into the parenchymal tissue. *Piscirickettsia salmonis* was abundant in most of the organs of fish that died during the study.

Out of all the mortalities, 12% of the fish had piscirickettsiosis alone, 76% had a mixed infection of *P. salmonis* and *A. salmonicida*, 8% had *A. salmonicida* alone, and no evidence of microorganisms was found in 4% of the mortalities. When comparing dead fish from the inoculated groups, 10 out of 19 of the IP challenged fish showed the presence of *P. salmonis* alone, whereas the fish challenged by PO and GS routes showed only mixed infections of *P. salmonis* and *A. salmonicida*.

Discussion

In this work, cultured *P. salmonis* was transmitted to freshwater-maintained Atlantic salmon by intraperitoneal injection, oral intubation and by application to the gill surface. *Piscirickettsia salmonis* was also detected in contact and non-contact cohabitants of fish belonging to each challenge group. In the directly challenged fish, as in the cohabitants, the presence of *P. salmonis* was easily detected by IFAT in organs such as liver and kidney, but was not very frequent in the gills or brain.

The first requisites for virulence in horizontally transmitted pathogens are the ability to survive in the aquatic environment and the ability to enter the host (Trust 1986). Fish pathogens gain entry to the fish via the gills and skin, and occasionally, via the oral route (Trust 1986). The oral route might be especially important in the case of *P. salmonis*, since natural outbreaks of SRS typically occur a few weeks after smolts are transferred to the sea (Branson & Nieto 1991; Cvitanich *et al.* 1991; Fryer *et al.* 1992). Smolts ingest and absorb large amounts of water to balance the osmotic loss of water after they are transferred to the marine environment (Evans 1993). Experimental infection of ayu, *Plecoglossus altivelis* Temminck & Schlegel, with *Vibrio anguillarum* in fresh water, by oral and anal intubation has been previously reported (Kanno, Nakai & Muroga 1989).

Based on the present results, the gills may also be important portals of entry during the natural transmission of SRS. Evidence that gills provide an effective and fast infection route has been demonstrated for several fish pathogens such as spring viraemia of carp virus (Ahne 1978), viral haemorrhagic septicaemia virus (Chilmonczyk 1980) and

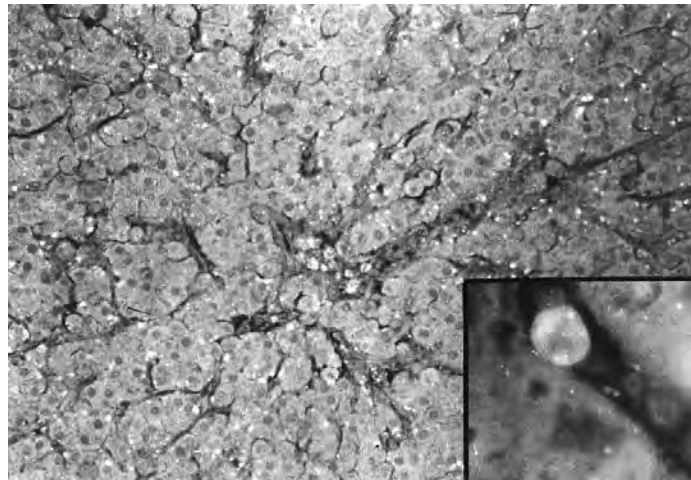


Figure 5 Atlantic salmon liver: electron microphotograph depicting haematogenous infection by *Piscirickettsia salmonis* 14 days post-oral challenge. The rickettsiae can be observed within the cytoplasm of leucocytes in the hepatic sinusoids (IFAT $\times 80$; inset $\times 800$).

A. salmonicida (Effendi & Austin 1995). *Aeromonas salmonicida* can enter Atlantic salmon through a range of sites including the mouth, gills, anus, flank, ventral surface and lateral line (Effendi & Austin 1995).

The importance of physical contact as a risk factor for transmission of SRS in fresh water has not been elucidated. Cvitanich *et al.* (1991) reported horizontal transmission of *P. salmonis* from IP inoculated coho salmon to contact cohabitants held in sea water or fresh water. Conversely, Garcés *et al.* (1991) found no evidence for horizontal transmission to contact cohabitants held in fresh water with IP challenged coho salmon. In the present study, piscirickettsial infection and mortalities developed in all contact and non-contact cohabitants in IP, PO or GS-challenged Atlantic salmon. In the IP and GS-infected groups, relative risk analysis of cohabitant fish showed a statistically higher risk of dying for cohabitants with physical contact (6.8) than for those without (2.5). *In vitro* experiments examining extracellular survival of purified *P. salmonis* (coho salmon isolates) showed that no infectious particles could be detected immediately after suspension in fresh water (Lannan & Fryer 1994). However, in salt water, infectious particles of *P. salmonis* were detected 10–15 days later (Lannan & Fry 1994). The poor capacity of rickettsial particles to survive in fresh water may explain the differences in the relative risk analysis caused by physical contact.

Within a tank of fish, there are several factors

promoting horizontal spread of established infections. For some diseases such as *Renibacterium salmoninarum* infection, an important epizootiologic factor is the close proximity between animals held for long periods (Murray, Evelyn, Beacham, Barner, Ketcheson & Prosperi-Porta 1992). Close contact with live or dead fish is also important in transmission of external parasites (Bakke, Harris, Jansen & Hansen 1992). Necrophagic behaviour could also be important for transmission of SRS, and thus, early extraction and appropriate disposal of mortalities might be important in the control of this disease. Additionally, the possibility that a biological vector may play a role in the transmission of *P. salmonis* in nature was suggested by the findings of a rickettsia antigenically similar to *P. salmonis* in sections of *Ceratomyxa gaudichaudii*, a common haematophagus ectoparasite of cultured salmonids in Chile (Garcés, Correa, Larenas, Contreras, Oyanadel, Fryer & Smith-Shuster 1994).

In non-contact cohabitants, transmission of the disease in fresh water is not easily explained. Cvitanich *et al.* (1991) reported abundant RLO-laden cells in the large intestine of coho salmon, and the authors hypothesized that the agent could be released through the faeces and survive for long enough to infect other fish. Furthermore, if viable rickettsia are present in the faeces, coprophagic behaviour may also be an alternative mechanism of transmission. Based on the favourable intravacuolar micro-environment created by rickettsial organisms contained inside the cell (Woldehiwet & Ristic

1993), it is also possible that *P. salmonis* surrounded by cellular debris, and mucous material from gill, gut or skin are protected from the hypotonic environment created by the fresh water. Cells observed carrying *P. salmonis* were mainly reticuloendothelial cells of the kidney, tubular epithelium and, sometimes, the glomeruli. Thus, this organism could also be released into the urine, and consequently, represent another factor involved in the horizontal transmission of SRS. However, further work is needed to clarify the mode of survival of *P. salmonis* in fresh water.

In the present study, mortalities of Atlantic salmon held in fresh water at 11 °C began after 12, 14 and 17 DPC in the orally, gill and IP challenged fish, respectively. Garcés *et al.* (1991) reported mortalities from 14 DPC in IP inoculated coho and Atlantic salmon held in fresh water at 10.5 °C. Another report showed that, in IP inoculated coho salmon held at 15 °C, mortalities started after 7 and 9 DPC in salt water, and after 10 and 11 DPC in freshwater aquaria (Cvitanich *et al.* 1991). Differences in the onset of mortalities may be explained by the water temperature, the species used, the size of the fish, the strain of *P. salmonis* used, the dosage of the inoculum and the presence of *A. salmonicida*.

Mortalities reaching 100% in IP inoculated Atlantic salmon have been previously reported (Garcés *et al.* 1991). In the present study, using a lower infectious dose, mortalities reached 50% in the experimental groups. Using the Cox proportional hazards survival analysis model, IP and GS-challenged fish had a lower cumulative probability of survival than PO infected fish ($P < 0.05$). No differences in cumulative probability of survival were observed between the IP- and GS-challenged fish, and no mortalities were observed in the unchallenged control group.

The IP route was previously reported as being effective in reproducing piscirickettsiosis in experimentally inoculated coho salmon and Atlantic salmon (Cvitanich *et al.* 1991; Garcés *et al.* 1991). Although the IP route does produce infection and IP infection through skin wounds is possible in nature, the oral and gill routes are more likely to represent the infection route in naturally infected fish. The pattern of infection in gill and orally infected fish resembles that observed in natural outbreaks of SRS (Branson & Nieto 1991; Cvitanich *et al.* 1991). The results of the present study have shown that the pattern of infection observed in the IP grouped fish is different from that observed in

orally, gill or cohabitant infected fish. A similar pattern of capsular infection was described following IP inoculation of Atlantic salmon and rainbow trout with *R. salmoninarum* (Bruno 1986). Although systemic piscirickettsiosis eventually developed in IP inoculated fish, the process of colonization of the host does not appear to resemble natural infection. Conversely, fish challenged orally or via the gill surface showed a systemic pattern of infection, with infected leucocytes commonly observed in blood vessels.

In the present experiment, *A. salmonicida* was detected in some fish. The organism was identified by morphologic characteristics and IFAT, and did not show cross-reactivity with the rabbit anti-*P. salmonis* antibody used in the analysis. It was not possible to establish the source of the *A. salmonicida* infection. Contamination of the water with *A. salmonicida* was unlikely since mortalities occurred only in fish infected with *P. salmonis* and *A. salmonicida* was detected only in one control fish. It could be argued that this pathogen may have been carried by some of the fish used in the present study. However, previous to the initiation of the experiments, a group of 60 fish from the stock were injected with prednisolone acetate to produce immunosuppression and detect carriers of common fish pathogens. None of the treated fish, nor any others from the same stock not involved in the present study showed clinical signs of disease, mortality or growth of *A. salmonicida* (Aqua Health Ltd, personal communication).

Mixed infections of *P. salmonis* with other pathogens have been reported previously (Cvitanich *et al.* 1991). The presence of *R. salmoninarum*, the causative agent of bacterial kidney disease (BKD), along with *P. salmonis* infection, has been described in sea water and fresh water outbreaks (Cvitanich *et al.* 1991; Gaggero *et al.* 1995). Additionally, a microsporidian protozoa (*Enterocytozoon salmonis*) has been described in salmonids with piscirickettsiosis (Cvitanich *et al.* 1991). Rickettsial organisms infecting leucocytes may produce an immunosuppressor effect on the host that can allow other pathogens to proliferate (Woldehiwet & Ristic 1993). The presence of *P. salmonis* within leucocytes and the double infection observed in some challenged fish suggest an immunosuppressive effect of *P. salmonis* that may have played a role in the capacity of *A. salmonicida* to infect and proliferate in the host.

In the present study, oral and gill routes were shown to be likely portals of entry for *P. salmonis*

infection. The importance of physical contact as a risk factor for the horizontal transmission of SRS was also confirmed, although physical contact was not necessary for horizontal transmission in fresh water. The sequential IFAT study showed that orally and gill-infected fish and their cohabitants showed a similar systemic pattern of infection, which was different from the capsular (serosal) infection pattern observed in IP inoculated fish. The presence of *P. salmonis* in the cytoplasm of epithelial cells of the renal tubules indicates that elimination of this pathogen through urine may be possible. Additionally, the presence of *P. salmonis* within leucocytes and the double infection (*P. salmonis*–*A. salmonicida*) observed in some of the fish exposed to this pathogen suggest an immunosuppressive effect of *Piscirickettsia salmonis* that may predispose the host to infections with other pathogens.

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Status and future perspectives of vaccines for industrialised fin-fish farming



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ABSTRACT

Fin fish farming is developing from extensive to intensive high industrial scale production. Production of fish in high-density growth conditions requires effective vaccines in order to control persistent and emerging diseases. Vaccines can also have significant positive impact on the reduced usage of antibiotics. This was demonstrated when vaccines were introduced in Norway for Atlantic salmon (*Salmo salar*) in the late eighties and early nineties, resulting in a rapid decline of antibiotics consumption. The present review will focus on current vaccine applications for farmed industrialized fish species such as Atlantic salmon, coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), ayu (*Plecoglossus altivelis*), cod (*Gadus morhua*), sea bass (*Dicentrarchus labrax*), gilt-head sea bream (*Sparus aurata*), yellowtail (*Seriola quinqueradiata*), great amberjack (*Seriola dumerili*), barramundi (*Lates calcarifer*), japanese flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), red sea bream (*Pagrus major*), rock bream (*Oplegnathus fasciatus*), seven band grouper (*Epinephelus septemfasciatus*), striped catfish (*Pangasianodon hypophthalmus*), channel catfish (*Ictalurus punctatus*) and tilapia (*Oreochromis niloticus*). This paper will review the current use of licensed vaccines in fin fish farming and describe vaccine administration regimes including immersion, oral and injection vaccination. Future trends for inactivated-, live attenuated - and DNA - vaccines will also be discussed.

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1. Introduction

About 600 aquatic species are farmed worldwide [21]. This includes both extensive low industrial scale production and more intensive high industrial scale production. The industrialised fish species are usually farmed at high-density under optimized growth conditions (light and temperature regimes) using intensive feeding with artificial feed. For these industrialised fish species the technological sophistication have increased in order to solve the many challenges that occur in an intensive production system [60]. Although nearly 2/3 of the fin fish production consist of extensive production of carps, the industrialisation of fin fish farming is expanding for both high and low value species (Fig. 1). Effective vaccines have probably been one of the most important factors for the growth and success of intensive salmonid farming systems. The introduction of water-in-oil (w/o) emulsion vaccines in Norway has demonstrated a significant impact on the reduction of antibiotics

from 50000 kg in 1987 to 1000–2000 kg in 1997, while at the same time the production increased from 50 000 tonnes to 350 000 tonnes [81]. The development of a sustainable industrialised aquaculture industry depends on the development and implementation of vaccines and vaccination regimes that makes the disease situation predictable and manageable under intensive production. This article will focus on current vaccine applications for farmed industrialized fish species and the future perspectives of vaccine technologies. It will focus on the licensed vaccines used in finfish farming, although the non-licensed ones can have a significant local impact on particular segments, as autogenous or other non licensed applications. However, as this use is normally not published and referred, it will not be covered in this review.

2. Vaccines for industrial scale fin-fish farming

Vaccines are available for more than 17 species of fish and protect against more than 22 different bacterial diseases and 6 viral diseases. Vaccines are available in more than 40 countries (Fig. 2). Although a large number of countries have some vaccines licensed, vaccination is mostly used in industrialized species in countries

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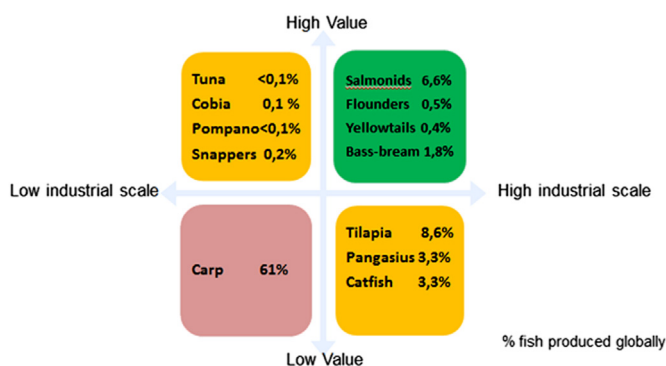


Fig. 1. Overview of industrialised farmed fish species categorised into low and high industrialised scale and high and low value of the product. Volume percentage of total fish produced is shown, based on FAO Fish stat+ 2011.

illustrated in Fig. 2. The large industrial scale vaccination was initially developed for vaccination of salmonids, but have now been implemented for several species.

In major fish producing countries like China (the worlds largest producer of farmed fish covering 61% of the total Aquaculture production [21]), vaccination is not common practise, however, the intensive research on fish vaccines in China, shows promise for the development of new fish vaccines in China [99]. Current vaccine application is described for the most important species/groups below. Fish vaccines are produced by a wide range of companies, of which major producers are listed in Table 1.

3. Vaccine administration

Administration of vaccines is either performed orally through feed, by immersion in diluted vaccine suspensions or by injection

via the intraperitoneal (w/o based vaccines) or intramuscular (DNA vaccines) route. In general, the level and duration of efficacy are highest with the injection method, however, so is also the handling and stress of fish. Fry which are too small to be injected are usually vaccinated by immersion or by the oral route.

3.1. Injectable vaccines

Injection vaccination has been important for disease protection of salmon and trout during the production cycle to harvest. In late 1980s the salmonid vaccine market primarily used water-based vaccines against pathogens such as *Listonella anguillarum* (*L. anguillarum*), *Vibrio* (*Aliivibrio*) *salmonicida* (*V. salmonicida*) and *Yersinia ruckeri* (*Y. ruckeri*). However, the introduction of *Aeromonas salmonicida* (*A. salmonicida*) into the Norwegian salmon farming industry resulted in high mortality for which water-based furunculosis vaccines were not sufficiently protective. The introduction of oil-based vaccines in the early 1990s resulted in significant reduction of furunculosis outbreaks and subsequently also a reduction of the use of water-based injection and immersion vaccines as described by Sommerset et al. 2005. The high efficacy and long duration of protection that the water-in-oil emulsion (w/o) adjuvants induce have been essential for the growth of salmonid aquaculture. There are many types of oil-containing emulsions such as oil-in-water (o/w), water-in-oil (w/o), water-in-oil-in-water (w/o/w) and oil-in-water-in-oil (o/w/o) emulsions, for which w/o is primarily used for fish vaccines due the formulations ability to induce long term protection [1]. The introduction of these effective w/o vaccines makes it possible to harvest fish at a larger size, resulting in improved economy for the farmers. W/o based vaccines have been related to injection-site reactions causing reduced growth and downgrading of Atlantic salmon (*Salmo salar*) at harvest [57]. However, this problem has in general been reduced. Studies also show that the inflammatory reactions vary within the

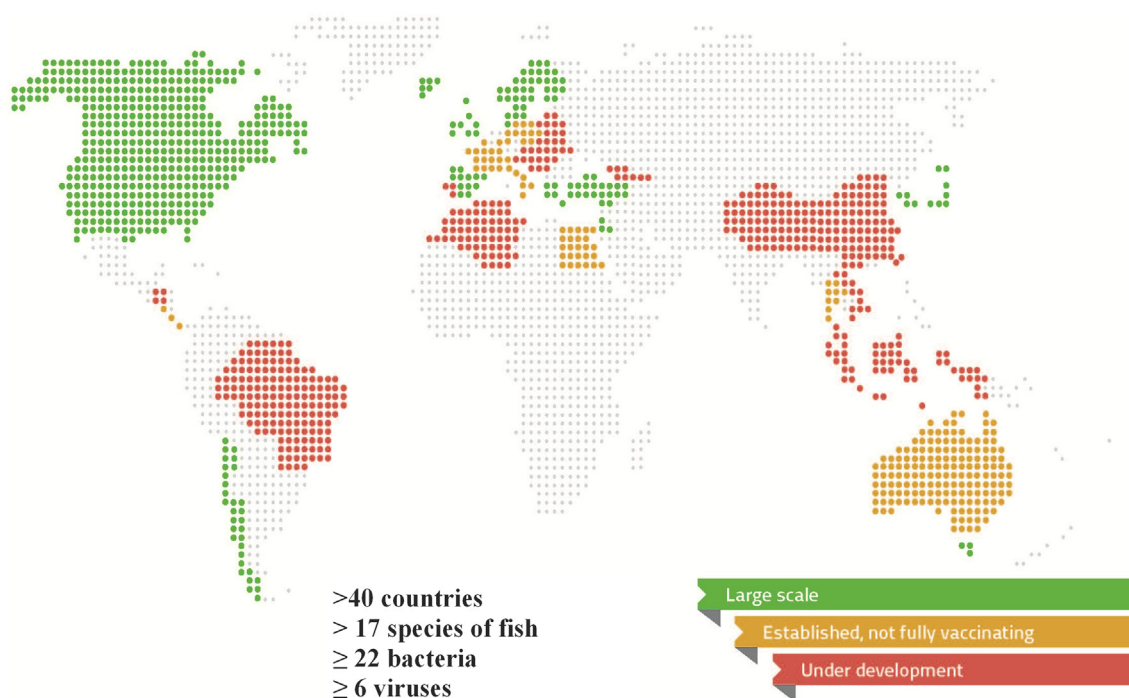


Fig. 2. A categorisation of the countries according to the use and implementation of fish vaccination. Green shows countries where vaccination is commonly used. Yellow are countries where vaccination is used, but not fully implemented. Red are countries where fish vaccination is under development. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Major producers of licensed fish vaccines listed in alphabetical order.

Company	Origin
Agrovet	Chile
Centrovet	Chile
DaeSung Micro. Bio. Lab	Korea
Dainippon	Japan
Goryo B&P	Korea
Green Cross	Korea
Hipra	Spain
JungAn Vaccine	Korea
Komi Pharm	Korea
Korea BNP	Korea
Kyoritsu	Japan
MSD	USA
Nisseiken	Japan
Novartis	Switzerland
PHARMAQ	Norway
Pfizer	USA
Tecnovax	Argentina
Recalcine	Chile
Veterquimica	Chile

salmonid species [64]. In other species side effects are usually less severe such as in sea bass (*Dicentrarchus labrax*) [2], cod (*Gadus morhua*) [55], turbot (*Scophthalmus maximus*) [7,9] and yellowtail (*Seriola quinqueradiata*) [32]. The vaccination procedure has usually been performed by vaccination teams manually performing vaccination of anesthetized fish. A skilled vaccinator is capable of vaccinating up to 3500 fish per hour [70]. However, the high labour costs for manual vaccination have in the last decade resulted in development of automated vaccination machines that are capable of handling up to 20 000 fish per hour [56].

DNA vaccines are administered by intra muscular injection such as DNA vaccine against infectious hemorrhagic necrosis, IHN.

3.2. Immersion vaccines

Commercial immersion vaccines are primarily in the form of formalin inactivated bacterial suspension or as live bacterial vaccines. The vaccination procedures for inactivated antigens are either by short dip in concentrated antigen suspension or a longer duration of exposure in a more diluted bath. Many inactivated immersion vaccines are used at 1/10 dilution of a concentrated vaccine suspension. The exposure duration is usually between 30 and 60 s. Early studies [87] using radio labelled *A. salmonicida* showed that 1/10 dilution of vaccine suspension gave no significant difference in antigen uptake in rainbow trout between 5 s and 10 min exposure time. Furthermore, a 1/100 dilution for two hours exposure duration resulted in significantly higher uptake compared to 100 s and 10 min exposure time. Inactivated immersion vaccines are also administered by bath directly in the holding tank for 30 min using 1/500 dilution. This is usually performed by reducing the water level in the tank and increasing oxygenation. The reduced handling of fish for bath vaccination compared to dip makes it both less labour intensive and less stressful for the fish. Several techniques have been described for improving immersion vaccine uptake such as hyperosmotic dip [38,90], ultrasound mediated uptake [26–28] and multiple puncture instrument [67]. Although, these methods have showed increased uptake of vaccine components during vaccination, none have yet gained any widely commercial use in the fish vaccination industry.

For diseases occurring in the fry stage such as rainbow trout fry syndrome and infectious pancreas necrosis (IPN), it is important to vaccinate as early as possible. However, efficacy is dependent on the development of immunocompetence in the vaccinated fish. A

study by Amend and Johnson [4] showed that coho salmon (*Oncorhynchus kisutch*) below 1 g had a delayed and poor response to vaccination, from 1 to 2 g improved onset was achieved but protection declined after 3–4 months. Long term duration of protection was only achieved on sizes above 2 g.

Live vaccines used for catfish production in USA against *Edwardsiella ictaluri* (*E. ictaluri*) and *Flavobacterium columnaris* (*F. columnaris*), consists of frozen vials where each vial of vaccine is sufficient to vaccinate 3.4 kg of catfish in approximately 20 L of water. The vaccine has shown to be efficacious in channel catfish (*Ictalurus punctatus*) fry as early as 7–30 days post hatching [46,78].

3.3. Oral vaccines

Oral vaccination with antigens included in the feed is in principle the ideal method of vaccine delivery. Such products are usually sold as suspensions for either directly coating the feed with antigen or mixing antigen into the feed during production. The efficacy of oral vaccines is dependent on antigen content in the feed [30] resistance against gastric degradation and antigen adsorption/uptake in the gut. MSD have a patented encapsulation technology which is used in their oral vaccines, while Centrovet Laboratories uses a patented (developed by Advanced BioNutrition Corp.) MicroMatrix™ Targeted Delivery Systems (TDS) for the prevention of piscirickettsiosis also called salmon rickettsial septicemia (SRS), and infectious salmon anaemia (ISA). Three companies have licensed oral vaccine to prevent disease caused by *Lactococcus garvieae* (*L. garvieae*) in 100–400 g *Seriola* sp. in Japan. Oral vaccines can be used for primary vaccination or as a booster vaccine to improve protection against long-lasting endemic diseases.

3.4. Vaccine regimes

For many species, a vaccination regime has been developed in order to keep fish protected all through the production cycle. Vaccination can be performed with up to three administration time points, usually starting with dip-vaccination, followed by booster vaccination (orally or dip/bath) and, finally, an injection vaccination. After injection of w/o based vaccines, subsequent vaccinations are not usually performed. However, oral booster vaccination against piscirickettsiosis after injection vaccination is performed in Chile. Booster vaccination is often performed by bath or by oral delivery in order to reduce the work involved in administering vaccine and, consequently, also the stress on the fish. Immersion (dip or bath) vaccination regimes have been described for sea bass against vibriosis and pasteurellosis [30] or by oral delivery as described in the Vaccination Guide [40].

4. Licensed vaccines for industrialised fin fish production

4.1. Salmonids

The high value industrialized salmonid species in global aquaculture are Atlantic salmon, coho salmon, rainbow trout (*Oncorhynchus mykiss*) and ayu (*Plecoglossus altivelis*). The type of disease challenges in these species differs, both in severity and occurrence in separate regions. Consequently this reflects the use of different types of vaccines listed in Table 2, as well as the vaccination strategies applied.

4.1.1. Atlantic salmon and coho salmon

Atlantic salmon is by far the most significant salmonid species in global aquaculture today, both in terms of value as well as scale of production, which occurs primarily in Norway, Chile, Canada, Scotland, Ireland, the Faroe Islands, Iceland and Australia. The total

Table 2
Summary of antigens in licensed vaccines, commercially available for anadromous and freshwater salmonids.

Species	Region	<i>A. salmonicida</i>	<i>L. anguillarum</i>	<i>V. salmonicida</i>	<i>V. ordalii</i>	<i>M. viscosa</i>	<i>Y. ruckeri</i>	<i>P. salmonis</i>	<i>R. salmoninarum</i>	<i>F. columnaris</i>	<i>L. garvieae</i>	IPNV	ISAV	PDV	IHN ^a
Atlantic salmon	Norway and Faroe islands	Inj	Inj	Inj		Inj	Imm					Inj	Inj	Inj ^a	
	Chile	Inj			Inj		Imm	Inj, Ora				Inj, Ora	Inj, Ora		
	UK/Ireland	Inj					Imm					Inj		Inj	
	N.America	Inj, Imm		Inj			Imm		Inj				Inj		Inj ^a
Rainbow trout	Norway	Inj	Inj, Imm	Inj			Imm								
	Europe	Inj	Imm, Inj, Ora				Imm, Inj, Ora				Inj				
	(exclusive Norway)														
	Chile	Inj					Imm	Inj, Ora				Inj, Ora			
	N. America	Inj, Imm			Inj		Imm		Inj	Imm					
	Japan	Inj	Imm				Imm								
Coho salmon	Chile	Inj			Inj		Imm	Inj, Ora				Inj, Ora	Inj, Ora		
Ayu	Japan		Imm				Imm								

Imm = Immersion vaccine, Ora = Oral vaccine, Inj = Injectable vaccine.

^a No injectable PD vaccine in Faroe Islands.

^a DNA vaccine.

global production of Atlantic salmon in 2011 was 1 619 200 tonnes [47].

Several licensed fish vaccines for Atlantic salmon and coho salmon are commercially available. Licensed vaccines against the following diseases are known; furunculosis (*A. salmonicida*), vibriosis (*L. anguillarum* and *Vibrio ordalii* (*V. ordalii*)), cold-water vibriosis (*V. salmonicida*), winter-ulcer (*Moritella viscosa* (*M. viscosa*)), yersiniosis- Enteric Red Mouth (ERM (*Y. ruckeri*)), piscirickettsiosis (*Piscirickettsia salmonis* (*P. salmonis*)), bacterial kidney disease (BKD) (*Renibacterium salmoninarum* (*R. salmoninarum*)), infectious pancreas necrosis (IPN virus), pancreas disease (Salmonid Alpha virus), infectious salmon anaemia (ISA virus) and infectious hemorrhagic necrosis (IHN virus). Most of the vaccines are w/o based vaccines administered by intra-peritoneal injection. However, different vaccination regimes are applied dependent on the regional prevalence of specific diseases.

The most comprehensive w/o based injectable vaccine for Atlantic salmon is composed of seven different antigens protecting against furunculosis, vibriosis, cold-water vibriosis, winter-ulcer, IPN and ISA. However, in Norwegian salmon farming more than 95% of Atlantic salmon smolts, equivalent to approximately 300 million fish on an annual basis per August 2012, were vaccinated with six-component w/o based vaccines containing antigens protecting against furunculosis, vibriosis, cold-water vibriosis, winter ulcer and IPN [73]. The vaccine is administered at the freshwater site as a single injection. Two to three weeks or at least 230° days prior to this, approximately 30% of Atlantic smolts were vaccinated with a monovalent w/o based vaccine against PD, a condition which causes major losses in the Norwegian industry [5].

In the Faroe Islands, vaccination against ISA in Atlantic salmon was included as part of the national contingency plan against this disease [17]. Today more than 90% of salmon smolts in the Faroe Islands are vaccinated with a seven-component vaccine.

In the Atlantic salmon industries in Scotland and Ireland combined w/o based vaccines against furunculosis and IPN are most commonly in use. In addition, a large proportion of pre-smolts are vaccinated with a single, w/o based monovalent PD- vaccine, prior to vaccination with the divalent vaccine.

In Canada, Atlantic salmon is produced both on the east Atlantic coast and the west Pacific coasts. The different disease challenges, the large distances between the coasts and also within the east coasts warrant different vaccine strategies. East coast producers typically vaccinate against furunculosis, vibriosis and cold-water vibriosis with w/o based vaccines. In addition a multivalent w/o based vaccine also including ISA-virus is commonly in use. On the west coast it is routinely vaccinated against the same bacterial diseases as on the east coast. In addition, a separate intra muscular injection with a monovalent saline based DNA vaccine against IHN, is commonly used. This is so far the only licensed DNA vaccine for aquaculture. The USA has a small Atlantic salmon industry with presence on both coasts (Maine and Washington States). Vaccine use reflects use on the Canadian side on respective coasts, however there is no licensed IHN-vaccine available in the Washington State. In the fresh water fry stage Atlantic salmon are commonly vaccinated against ERM and furunculosis. In USA and Canada there is also an attenuated *Arthrobacter*-based commercial vaccine against BKD available.

In Chile, vaccines designed for Atlantic salmon have been developed over the past 11 year period. The first w/o based vaccine in use was a monovalent vaccine against IPN followed by divalent w/o based vaccines against furunculosis and IPN. Further, trivalent vaccines including antigen protecting against *V. ordalii* were licensed a few years later followed by a four-component vaccine also protecting against piscirickettsiosis in 2009. No ISA-vaccines were licensed before 2007 when ISA hit the industry and led to a

serious downturn in Atlantic salmon industry production. Different vaccines were licensed in Chile and today all Atlantic salmon are vaccinated with w/o based vaccines. Currently, the most common vaccination regime for Atlantic salmon includes injection with five-component vaccines against furunculosis, vibriosis, piscirickettsiosis, IPN and ISA. Some farmers have also included oral vaccination of Atlantic salmon at 1–1.5 kg against piscirickettsiosis as part of their vaccination regime. At the early freshwater stage mortality from IPN is frequently seen [16] and as part of their vaccination program, some farmers apply IPN-immersion vaccines at 2–3 g size and/or IPN-monovalent vaccines administered by injection to 8–10 g fish.

The current vaccination regime for coho salmon in Chile is vaccination against IPN and piscirickettsiosis with w/o based divalent vaccines.

In Australia (Tasmania), the production of Atlantic salmon in 2011 were 36 000 tonnes [47]. There are no commercial, registered vaccines available to the industry except four different autogenous vaccines for fish. Amoebic gill disease (AGD) is the most serious health problem in Atlantic salmon cultured in Tasmania [10].

In Japan, the production of salmonids (ayu, coho and trout) were 30 000 tonnes in 2010 [24]. The fish are vaccinated by immersion against *L. anguillarum* serotype J-O-1 and J-O-3 [65]. The serotype J-O-1 is European serotype O2, and J-O-3 is equivalent to serotype O1 [94].

4.1.2. Rainbow trout

The production of rainbow trout can be differentiated into large trout production in seawater/brackish water and freshwater pan sized/inland production. The primary producing areas are in Europe, North America, Chile, Japan and Australia. Chile is currently the largest producer [21].

The diseases affecting rainbow trout in seawater production of large trout versus freshwater rainbow trout production differ in the context of vaccines available and vaccination regimes most frequently used. Licensed vaccines against the following diseases are known; furunculosis (*A. salmonicida*), vibriosis (*L. anguillarum* and *V. ordalii*), winter-ulcer (*M. viscosa*), yersiniosis-ERM (*Y. ruckeri*), lactococcosis (*L. garvieae*), flavobacteriosis (*F. columnare*), piscirickettsiosis (*P. salmonis*) and IPN. Most of the vaccines for large rainbow trout are w/o based injectable vaccines compared to mainly water-based vaccines used for freshwater rainbow trout and at early fry stage.

4.1.2.1. Large rainbow trout production in seawater. The majority of the global large trout production takes place in Norway, Finland, Sweden, Denmark and Chile. The total global harvest of farmed large trout in 2011 was 316 700 tonnes [47].

In Norway rainbow trout are routinely vaccinated with w/o based vaccines against furunculosis and vibriosis. However, winter-ulcer in trout is common and multivalent vaccines including a *M. viscosa* component are often preferred.

In Finland, Sweden and Denmark vaccination against furunculosis and vibriosis with w/o based vaccines is a standard vaccination regime for large rainbow trout. In Denmark, most rainbow trout fry are primarily immersion vaccinated against yersiniosis at 3–5 g. Due to an increased prevalence of yersiniosis in brackish farm sites in the Baltic Sea over the past years [84], simultaneous vaccination by injection of a water-based ERM-vaccine and a w/o based furunculosis/vibriosis vaccine is more commonly seen.

In Canada, the rainbow trout in salt or brackish water are produced on the east coast. The fish are vaccinated against furunculosis and/or vibriosis prior to transfer to the marine environment. Both w/o based multivalent and monovalent water based vibrio vaccines are in use. In the fry stage the trout is commonly vaccinated against ERM with water-based vaccines.

In Chile the use of vaccines for rainbow trout began in mid nineties' with immersion vaccination against *Y. ruckeri* and *Flavobacterium psychrophilum* (*F. psychrophilum*). Vaccination against yersiniosis is still a common practice in 2012 whilst vaccines against flavobacteriosis are no longer available. By the late nineties' vaccines with a water-based *P. salmonis* component against piscirickettsiosis was licensed, shortly followed by an introduction of a w/o based vaccine. The current vaccination regime for large rainbow trout is vaccination to protect against IPN and piscirickettsiosis with w/o based divalent vaccines. Some farmers use a trivalent w/o based vaccine against vibriosis, IPN and piscirickettsiosis. In addition, some farmers also vaccinate with an oral vaccine protecting against piscirickettsiosis at 1–1.5 kg. At the early freshwater stage of rainbow trout, as for Atlantic salmon, mortality due to IPN is seen, and as part of their vaccination program, some farmers apply IPN-immersion vaccines to rainbow trout at 2–3 g size and/or IPN-monovalent vaccines administered by injection to 8–10 g fish.

4.1.2.2. Freshwater rainbow trout production. Fresh water rainbow trout is the most commonly farmed salmonid throughout the mainland Europe where each country shares a common disease profile due to the nature of the industry which imports/exports live fish regularly and the general farming practises which are employed.

The two major bacterial disease challenges to freshwater rainbow trout production in Europe are yersiniosis (also called enteric redmouth disease) and flavobacteriosis (caused by *F. psychrophilum*) known as rainbow trout fry syndrome (RTFS). Although flavobacteriosis is a significant disease threat to the industry in most rainbow trout producing countries there are no fully licensed immersion- or injectable vaccines available. Furunculosis in freshwater rainbow trout production is frequently described, and recently vibriosis caused by *L. anguillarum*, found in Turkish freshwater rainbow trout was reported to rapidly spread amongst farms [86]. Lactococcosis caused by *L. garvieae* is seen in several rainbow trout producing countries especially in southern Europe [95], and documented as a major bacterial disease in Iran, a significant producer of trout estimated to be the largest in the Middle East and equal to Turkey.

Water-based injection- and immersion vaccines against yersiniosis and vibriosis in rainbow trout are licensed in several European countries as well as oral vaccines against the same diseases [34]. As a general regime, most rainbow trout fry are vaccinated against yersiniosis by one immersion at 3–5 g size only, during the production cycle. However, a booster vaccination should always be considered. Vaccination using a w/o based injectable vaccine against lactococcosis is part of a specific vaccine regime in some European countries.

Rainbow trout is produced in North America, both in the USA and Canada. The main area of food fish production in the USA is Idaho, with around 70% of the volume, typically producing 500–600 g portion sized trout [96]. Ontario is Canada's largest food trout production region, targeting mostly 1 kg rainbow trout [22].

Major bacterial endemic diseases in North American rainbow trout production include ERM and flavobacteriosis, furunculosis and BKD. Water-based immersion vaccines are commercially available against the three diseases first listed, and fish are typically immersed once or twice from 2 to 3 g and booster vaccinated at around 5 g. A live attenuated *Arthrobacter*-based injection vaccine is available against BKD, injected intraperitoneally to fish of 10 g or more. An overview over licensed, commercial fish vaccines in Canada and USA are publically available online [8].

The major viral disease of concern is IHN, which is endemic in the western part of North America. The DNA vaccine APEX-IHN is licensed for salmonids, however it is not commonly used in trout.

4.2. Marine species

4.2.1. Cod

Most of the farmed cod is produced in Norway. The production in 2011 was 15 000 tonnes [22]. Atypical furunculosis and vibriosis are the most typical bacterial diseases in cod today. The prevalence of francisellosis caused by *Francisella noatunensis* (*F. noatunensis*) in Norwegian cod farming is significantly reduced since 2008 and no vaccines are currently available. Water-based vibrio vaccines for immersion are applied according to a standard regime recommending vaccination at 1 g fish size followed by booster vaccination at 5 g. A w/o based injection vaccine against vibriosis and atypical furunculosis is available in Norway for vaccination of cod at minimum 30 g size Table 3.

4.2.2. Sea bass and sea bream

The Mediterranean countries are the leading producers of farmed European sea bass (*Dicentrarchus labrax*) and gilt-head sea bream (*Sparus aurata*). The production was 129 000 tonnes of sea bass and 161 000 tonnes of sea bream in 2011 [48]. The bacterial diseases most often found are vibriosis (*L. anguillarum*) and pasteurellosis (*Photobacterium damsela* (*P. damsela*)). The prevalence of vibrio infections in sea bass is higher compared to sea bream. Since the mid nineties a recommended injection vaccination regime applied for sea bass has been successful. A combined water-based immersion vaccine against vibrio and pasteurellosis is recommended at 1 g and re-vaccination at 5 g followed by injection of a combined w/o based vaccine at minimum 15 g size. Monovalent water-based vibrio and pasteurella vaccines for immersion are available in selected markets (Table 3).

4.2.3. Yellowtail and great amberjack

Seriola species as yellowtail (*Seriola quinqueradiata*) and great amberjack (*Seriola dumerili*) are mainly produced in Japan. The production was 139 000 tonnes in 2010 [20]. Immersion vaccine against *L. anguillarum* J-O-3, is available for fish at 1–3 g. Vaccination started with the use of oral vaccine against *L. garvieae* in 1997. The vaccine is recommended used from 100 to 400 g. Later through a series of mono and divalent vaccines, trivalent water-based injection vaccines containing iridovirus, *L. anguillarum* (J-O-3) and *L. garvieae* or *L. garvieae*, *L. anguillarum* and *Streptococcus dysgalactiae* (*S. dysgalactiae*) are now used from 10 g in weight. Also two w/o based vaccines, one divalent and one trivalent, recommended for fish from 30 g up. The trivalent w/o based vaccine protects against *P. damsela*, *L. garvieae* and *L. anguillarum* (J-O-3) [66]. Vaccination of yellowtail against pasteurellosis, was not reported to cause any significant side effects [31] (Table 3).

4.2.4. Asian sea bass/barramundi

Asian sea bass or barramundi (*Lates calcarifer*) is produced in many Asian countries and in Australia. The production volume is 66 000 tonnes in 2010, of which 1/3 is from Malaysia [21]. *Streptococcus iniae* (*S. iniae*) causes mortality particularly in bigger fish. A vaccine against *S. iniae*, is licensed in Singapore and Indonesia (Neil Wendover, pers com), the vaccine is inactivated water-based and can be administered by immersion or injection [59]. Vaccine for iridoviral disease is also available in Singapore. No side effects have been reported in connection with vaccination of Asian sea bass. Other diseases reported are tenacibaculosis (caused by *Tenacibaculum maritimum* (*T. maritimum*)), and viral neural necrosis (VNN) [98] (Table 3).

4.2.5. Flatfish (olive flounder and turbot)

Olive flounder (*Paralichthys olivaceus*) is produced in Japan, China and Korea. In Japan, injection vaccines against *S. iniae* are available and recommended for fish at 30–300 g [66]. In Korea, an immersion vaccine is available against *Edwardsiella tarda* (*E. tarda*) [72]. However, injection vaccination is more used. Through successive development of monovalent injection vaccines, a trivalent water-based vaccine against *S. iniae*, *Streptococcus parauberis* (*S. parauberis*) and *E. tarda* is developed and approved by the Korean animal, plant & fisheries quarantine & inspection agency. The development is now moving towards five valent vaccines including *L. anguillarum* and *T. maritimum* [42]. In China, there is a vaccine available for *Vibrio* sp and *E. tarda* (Dr. Jie Huang, pers com).

Production of farmed turbot is geographically wide spread in Europe with the largest production volumes in Spain. According to the Business Association of Marine Aquaculture Producers (Apro-mar) the production reached 7755 tonnes. A range of different bacterial pathogens challenge the European turbot industry, e. g. *E. tarda*, *L. anguillarum*, *T. maritimum*, *S. parauberis* and atypical *A. salmonicida*. Prevalence of the different bacteria and disease challenges varies geographically. Vaccines against the bacteria listed above are developed, but to a variable degree available. Vaccines are also offered as autogenous products by vaccine manufacturers. There are commercial *L. anguillarum* vaccines available for immersion and injection administration [68,93], containing a mixture of serotypes O1 and/or O2a. However, a licensed w/o based vaccine (GAVA-3), marketed by Hipra Laboratories (Spain), is to our knowledge the only vaccine covering all three serotypes O1, O2a and O2b of *L. anguillarum* [95] (Table 3).

4.2.6. Other marine fish

Red sea bream (*Pagrus major*) is produced mainly in Japan and the production was 68 000 tonnes in 2010 [21]. Iridovirus causes disease in many marine species [65]. The inactivated red sea bream

Table 3
Summary of antigens in licensed vaccines, commercially available for marine and brackish water fish.

Species	Region	Atypical <i>A. salmonicida</i>	<i>E. tarda</i>	<i>P. damsella</i>	<i>L. anguillarum</i>	<i>S. iniae</i>	<i>S. parauberis</i>	<i>S. dysgalactiae</i>	<i>L. garvieae</i>	Iridovirus	VNNV
Cod	Norway	Inj			Inj, Imm						
Gilt-head sea bream	Greece, Turkey			Inj, Imm	Inj, Imm						
European sea bass	Greece Turkey			Inj, Imm	Inj, Imm						
Asian sea bass	Indonesia, Malaysia					Inj					
	Singapore									Inj	
Red sea bream	Japan									Inj	
Rock bream	Korea									Inj	
Yellowtail	Japan			Inj	Inj			Inj		Inj	
Great amberjack	Japan			Inj	Inj			Inj	Inj, Oral	Inj	
Striped Jack	Japan									Inj	
Grouper	Japan									Inj	
Turbot	Spain				Inj, Imm						Inj
Olive flounder	Korea, Japan		Inj, Imm			Inj	Inj				
Flatfish	China		Inj, Imm		Inj						

Imm = Immersion vaccine, Ora = Oral vaccine, Inj = Injectable vaccine.

iridovirus (RSIV) vaccine were first licensed for red sea bream in Japan and is administered by injection for 5–20 g fish [66]. The use of this vaccine has later been extended to *seriola* species that are injected at 10–100 g, striped jack at 10–70 g, and grouper at 5–50 g.

21 000 tonnes of rock bream (*Oplegnathus fasciatus*) was produced in Korea [21]. A recombinant vaccine is licensed for rock bream in Korea [72].

Recently a vaccine was licensed to protect seven band grouper (*Epinephelus septemfasciatus*) against VNN in Japan [69].

4.3. Fresh water species

4.3.1. Grass carp

The production of grass carp (*Ctenopharyngodon idellus*) is more than 4.3 mill tonnes globally, where of China produces 4.2 mill tonnes (FAO fish stat+ 2012).

A live attenuated vaccine has been launched for grass carp in China for spring viraemia of carp (Prof Huang Jie, pers. Com). It is also described by Ref. [99] that two inactivated vaccines against grass carp haemorrhage and *Aeromonas hydrophila* (*A. hydrophila*) have veterinary certificate of China, however with no real industrial applications when described in 2008 (Table 4).

4.3.2. Catfish (striped catfish (*pangasius*) and channel catfish)

The production of catfish is more than 3 mill tonnes globally [21]. The main countries are Vietnam (38%), China (26%), Indonesia (12%) and USA (7%). The major species are striped catfish (*Pangasianodon hypophthalmus*) and channel catfish.

Currently, two live attenuated vaccines have been developed and are commercially available for channel catfish in the U.S. for *E. ictaluri* and *F. columnare* [6] (Table 4). Bebak and Wagner reported that 9.7% of the fingerling production used one or both vaccines in 2009, while among the total industry 12.3% vaccinated against *Edwardsiella ictaluri*, and 17.4% vaccinated against *F. columnaris*. They also concluded that for striped catfish, there are no commercial vaccines available. However, an inactivated injection vaccine against *E. ictaluri* obtained a commercial license for Vietnam in 2013 (www.pharmaq.com), but large scale vaccination using the injection vaccine against *E. ictaluri* took place through an observation license during the last year [88]. There are no reports of side effects in connection with vaccination of catfish.

In addition to the diseases mentioned above, *A. hydrophila* was recently reported to cause severe mortality in catfish [74].

4.3.3. Tilapia

Tilapia is one of the main species produced in aquaculture world-wide. The total production of tilapia was 3.5 million tonnes in 2010 [21] and the main producing countries are China, Egypt, Indonesia, the Philippines, Thailand and Brazil. The most common

specie is *Oreochromis niloticus* with 2.5 million tonnes produced in 2010.

The main disease problem is streptococcosis, predominantly caused by infection of *Streptococcus agalactiae* (*S. agalactiae*), prevalent in temperate and tropical regions. This bacterial species, which constitutes at least 10 serotypes [80], is a well known pathogen towards humans and animals. In tilapia, three different serotypes are known to cause infections, serotype Ia [18], serotype Ib [97] and serotype III [85].

In tilapia production there is not a widespread use of vaccines. The relative low value of each individual fish at harvest makes it difficult for small fish farms to invest in effective vaccines. However, the trend in tilapia production is an increasingly intensive production and the emergence of larger farm companies with focus on reducing the use of antibiotics. Consequently, on-going development and testing of improved vaccines is an important aim for farmers and pharmaceutical companies.

A commercial vaccine for the control of *S. agalactiae* is available in Indonesia, Brazil and several Central American countries [15], in addition an inactivated w/o based vaccine has recently obtained approval from the authorities in Singapore against iridovirus (Genus Megalocytivirus) [23].

5. Future trends and technologies in fish vaccinology

To further support sustainable fin fish production in aquaculture there are many fish health related issues to be addressed which are important for fish welfare, profitability and the environment. These issues are the driving forces for the development of new vaccines and efficient vaccination programs against emerging and persistent diseases in aquaculture.

Developing fish vaccines is costly and time consuming, and it would probably not be profitable or realistic to develop vaccines against all pathogens identified. In order to increase availability of vaccines, contract production of different autogenous vaccines is one future scenario. However, the overall importance to be emphasized is vaccine product quality together with best practice vaccination and fish health management.

Most of the vaccines available are based on classical fermentation, cultivation or recombinant technologies. Future vaccines including inactivated vaccines, live attenuated vaccines and DNA vaccines will be reviewed in this section.

5.1. Adjuvants for inactivated antigens

For inactivated vaccines, adequate and long-lasting protection is only achieved when adjuvants are included to increase the potency of the formulation. In fish vaccinology, injectable w/o based adjuvants have remained the gold standard since the commercial introduction in the early 1980s. Although detailed knowledge

Table 4
Summary of antigens in licensed vaccines, commercially available for fresh water fish.

Species	Region	<i>E. ictaluri</i>	<i>F. columnare</i>	<i>S. agalactiae</i>	Iridovirus	Spring viraemia of carp
Channel catfish	USA	Imm ^a	Imm ^a			
Striped catfish	Vietnam	Inj ^b				
Tilapia	Brazil			Inj		
Tilapia	Costa Rica			Inj		
Tilapia	Indonesia			Inj		
Tilapia	Singapore				Inj	
Grass carp	China					Imm ^a

Imm = Immersion vaccine, Ora = Oral vaccine, Inj = Injectable vaccine.

^a Attenuated.

^b Inactivated injection.

about the mode of action for w/o-adjuvants remains to be fully elucidated, numerous studies have documented their ability to depot antigens at the site of injection [62,64], consequently upholding the pivotal contact between vaccine antigens and immune cells [19] to support sustained and robust pro-inflammatory [25,63] and Humoral responses [58].

In general, the injection vaccines presently on the market induce excellent protection against bacterial diseases such as vibriosis, furunculosis and winter ulcers [54]. However, there are some challenges associated with using w/o emulsions as adjuvants. First, the use of these adjuvants may cause adverse effects such as melanization, organ adhesions and autoimmunity [49,57]. A cardinal challenge has been to balance vaccine efficacy with the reactogenic nature manifested at the injection site. Vaccine improvements by formulation optimization and dose volume reduction have nevertheless drastically reduced the severity of side effects. The second main challenge that still remains is to develop vaccines that are equally potent in inducing immunity against intracellular pathogens as what has been documented for the bacterial diseases. For this purpose, w/o adjuvants are sub-optimal as they generally promote strong humoral immune responses [13]. Furthermore, modern fish vaccines for high value species such as salmonids are preferably multivalent containing antigens from several pathogens. The w/o adjuvant is an excellent platform for multivalent vaccines as numerous inactivated antigens may easily be incorporated, it does however demand for high titer production of pathogens. The latter may prove to be a key challenge for development of efficacious antiviral vaccines, since high antigen doses are advantageous for induction of protective immunity against viral diseases [61,89].

The progress in adjuvant technology for commercial injectable, inactivated fish vaccines have hitherto mainly been due to refinement rather than replacement of the existing w/o based adjuvants. The challenge of increasing the vaccine potency, predominantly against intracellular pathogens underlines the need to search for alternative adjuvants and delivery systems. During the last two decades a number of different adjuvants have been suggested. These adjuvants could be classified in two broad groups, namely soluble or particulate. The former group includes receptor ligands, such as Toll-receptors agonists, which have the advantage of directly binding intra- or extracellularly on innate and adaptive immune cells [33,71]. Thus, unlike for many of the established particle based adjuvants/delivery systems such as aluminium salts (alum), MF59 (o/w) and w/o-formulations, the cellular and molecular mechanisms of action of these soluble adjuvants are well defined and may prove useful for modulating responses to vaccination towards cellular immunity. In Atlantic salmon, synergistic antiviral responses have been reported after vaccination with unmethylated CpG oligodeoxynucleotides (ODN) and poly IC (synthetic double stranded RNA) against salmonid alpha virus (SAV3) [83]. The capacity of other TLR ligands, such as flagellin [39] and imiquimod [44] to induce immune responses have also been reported, but there are still few reports on the efficacy of these soluble adjuvants in protecting fish against disease. In studies on mammalian species, conjugation of TLR ligands to antigens has been shown to increase antigen potency by ensuring temporal and spatial co-delivery of immune modulators (TLR ligands) and antigens, which have been demonstrated to have several benefits over non-conjugated antigens [35].

Particle based adjuvants/delivery systems constitute a large number of biocompatible compounds that are partly or fully biodegradable, including particles made of poly (lactide-co-glycolide) acid (PLGA), chitosan, alginate, liposomes, virosomes, immune stimulating complexes (ISCOMs) and emulsions (w/o and o/w) [13]. Similar to what has been reported for soluble adjuvants, antigen-

particle association by absorption and entrapment is known to increase immune responses and vaccine potency/efficacy [36,77]. For non-emulsion particle based delivery systems, size is recognized as a highly important determinant for cellular uptake and antigen processing [43] where sub-micron particles have been demonstrated to support the MHC I/CTL pathway while microparticles, with release of antigens to the extracellular junctions, may support MHCII/Th2 characterized responses [41]. Particulate adjuvants that exists as single entities (e.g PLGA, ISCOMs, liposomes, virosomes etc) work as antigen vehicles and may thereby contribute to protect antigens from degradation, increase the cellular uptake and thus be in support of the depot concept [76]. This quality is specifically important to avoid premature antigen degradation during oral vaccine delivery [50,91,92].

Generally, the novel adjuvants and delivery systems that so far have been examined in fish seem to only induce transient immune responses, resulting in sup-optimal long term protection. We therefore advocate and renew the statement by Evensen et al. [19] who viewed it as unlikely that the w/o adjuvant will be replaced in the immediate future. However, to meet the requirements for improved antiviral responses to vaccination, future fish vaccines are expected to include refinements of the existing w/o-based adjuvant platform rather than fully replacing it.

5.2. Future trends – DNA vaccines

The DNA vaccine concept has been a promising candidate in the defence against pathogens, particularly pathogens such as viruses and intracellular bacteria that might require cell mediated antigen presentation for the correct activation of immunity. Indeed, aquaculture is one of the few areas where a DNA vaccine has actually entered the market. The IHNV DNA vaccine licensed for use in Canada has shown good protection against several IHNV virus strains [12]. Also DNA vaccines against other Rhabdoviruses show good effect, such as the results from research on a viral hemorrhagic septicemia DNA vaccine [53]. The G-protein of the *Rhabdoviridae* seems to induce an initial unspecific boost of the innate immune system, and hence can serve as an adjuvant and increase the effect of the DNA vaccine [45,79]. This might suggest that with a good adjuvant, DNA vaccines against pathogens outside of the *Rhabdoviridae* family that have not shown as promising results, might perform better [52]. Work on genetic adjuvants (DNA plasmids encoding proteins that are immune-stimulatory), as well as adjuvants that are a part of the vaccine, but not encoded on a plasmid, will be an important step towards better functioning DNA vaccines. Also improved transfection efficiency through new administration methods might serve to optimize the effect of DNA vaccination. Particularly this will be a challenge if multivalent DNA-vaccines are developed.

Another aspect is that the DNA vaccinated fish in some countries will be labelled as a Genetically Modified Organism (GMO). Public perception of GMOs as source of food, as well as restrictive legislation from the authorities on this matter might cause challenges for the aquaculture industry and will have to be addressed.

5.3. Future trends – live attenuated vaccines

The use of live vaccines is not a new concept, and the first known vaccine to protect humans against small pox was derived from a live virus used as a vaccine during the 1770s [82].

Despite the advantages of modified live vaccines compared to inactivated vaccine with respect to protection against difficult intracellular pathogens, few vaccines are on the market. Attenuated live vaccines survive and replicate within the host, which results in a strong cellular immune response that confers protection of long

duration. The live vaccine also has the ability to stimulate the humoral and mucosal immunity [11].

The major disadvantage of using modified live vaccines is related to safety concerns. The possible reversion to virulence means extensive work to document that this will not be a problem in practice is required before such a vaccine can be made commercially available. Several attenuated microorganisms have been shown to be unstable and revert to virulence [3,37,100]. A recent paper demonstrates that attenuated vaccines can recombine to form virulent field viruses in the poultry industry [51]. The important and comprehensive bio-safety assessments include biological safety to aquatic animals and environment as well as purity and efficacy of the vaccine. Presence of the pathogen in the natural environment and the ability to infect people are among the important considerations.

Traditionally, attenuation was achieved by multiple passages of the microorganism on appropriate growth medium which resulted in random mutations that lead to attenuation. Chemical or physical mutagenesis results in random mutation(s) in the pathogen. An environmental bacterium mimicking the antigenic structure of the pathogen is also used (*Arthrobacter davidanieli*) to protect against BKD caused by *Renibacterium salmoninarum* [14,75]. Novel molecular methods enable the development of GMOs targeted to specific genes. GMOs constructed by genetic engineering display an advantage over random mutagenesis, enabling a more targeted safety testing. Another approach is the introduction of foreign genes into a live vaccine strain which expand the protective activity against more diseases. However, attention must be paid to the biological safety assessments as the recombinant hosts acquire new properties. There are regulatory aspects related to the use of live GMO vaccines as they are released into the environment. The regulatory restrictions in many countries are significantly stricter than those for the release of conventional live vaccines. This is in contrast to biological safety considerations where defined genetic modifications allow for much better control and safety assessment than random, mostly unknown mutations in a conventionally attenuated vaccine [29].

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A history of fish vaccination Science-based disease prevention in aquaculture



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ABSTRACT

Disease prevention and control are crucial in order to maintain a sustainable aquaculture, both economically and environmentally. Prophylactic measures based on stimulation of the immune system of the fish have been an effective measure for achieving this goal. Immunoprophylaxis has become an important part in the successful development of the fish-farming industry. The first vaccine for aquaculture, a vaccine for prevention of yersiniosis in salmonid fish, was licensed in USA in 1976. Since then the use of vaccines has expanded to new countries and new species simultaneous with the growth of the aquaculture industry. This paper gives an overview of the achievements in fish vaccinology with particular emphasis on immunoprophylaxis as a practical tool for a successful development of bio-production of aquatic animals.

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1. Introduction

Compared to animal husbandry fish farming is a relatively new method for bioproduction in many countries. When diseases appeared in aquaculture operations with fish, antibiotics or chemotherapeutics were used for disease treatment, and even for disease prevention.

The first report of disease prevention using vaccines is probably by Snieszko et al. [1] who published a paper in 1938 about protective immunity in carp immunized with *Aeromonas punctata*. Their paper was written in Polish which reduced – unfortunately – the availability elsewhere in the world. The first report in English was written by Duff in 1942, who showed protection against *Aeromonas salmonicida* in trout immunized by parenteral inoculation and by oral administration [2].

During the first 20–30 years after World War II there are only a limited number of reports about disease prevention in aquaculture by vaccination. The most important reason for the disinterest in immunoprophylaxis in these years was probably the availability of antimicrobial compounds. A study by Snieszko and Friddle [3] concluded that chemotherapy with sulfamerazine was superior to oral administration of a vaccine for the control of furunculosis. It was not until the seventies that vaccines were applied in

commercial aquaculture. In aquaculture, the years after World War II are therefore called “the era of chemotherapy” in a paper by Evelyn [4].

2. Vaccinology

The word “vaccination” originated from the work of Jenner who inoculated a boy with infectious material from cowpox in order to induce immunity to smallpox [5]. He called the process “vaccine inoculation”, later “vaccination”. Later Louis Pasteur suggested that the word “vaccination” should be a general word for preventive inoculation of microorganisms as a tribute to the work of Jenner (cited in Ref. [6]).

The word “vaccinology” is of more recent vintage. Salk and Salk [7] introduced vaccinology in 1977 to describe the interdisciplinary dimension of disease prevention based on microorganisms stimulating the immune system to prevent infectious diseases in individuals and populations.

Vaccinology includes different disciplines. Immunology is one of them. During the last 150 years there have been several studies of fish immunology and biology with relevance to vaccinology, reviewed by Evelyn [4], Van Muiswinkel [8] and Van Muiswinkel and Nakao [9]. The background of the scientists has varied, including biology, anatomy, hematology, physiology, ichthyology, microbiology, pathology, fish diseases and others. Research on induction of humoral antibodies in immunized fish was published before World War II [10,11], and continued with studies of

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immunoglobulins, lysozyme and complement as well as the cellular basis of the immune response [12–16]. Fish was also included in studies of comparative and developmental immunology in order to get a better understanding of evolutionary development of the immune system [15].

Different fish species, including carp and salmonid fish, were included in the immunological studies, and factors of importance in vaccinology, like temperature and other environmental factors, were also studied [17,18]. The role of adjuvants in fish is important both for the progress in immunology and vaccinology. Ambrosius and Lehmann [19] found that adjuvants like aluminum hydroxide and Freund's adjuvant increased the quantity of immunoglobulins, the former slightly and the latter significantly.

The method of administration was also studied. Trout was found to produce a good immune response when injected with antigens from *A. salmonicida* [12]. However, parenteral introduction of antigens seemed to be economically prohibitive and might even be harmful to the fish. Oral immunization was therefore considered to be the only way for practical disease prevention [20].

This assessment was also influenced by results from studies on humans and terrestrial animals. The successful vaccination against poliomyelitis in humans [21] and Newcastle disease in poultry [22] by oral administration probably influenced priorities and direction of research of scientists working with fish immunology and vaccinology. Industrial production of poultry and fish had similarities and scientists working with different vertebrates shared information.

Experience from the poultry industry was probably also the basis for spray vaccination of fish as a method for administration of antigens. Spray vaccination of fish was given a US patent [23], but the method was not widely used in the field.

Successful vaccination could also be performed by dipping the fish for a short period in a diluted vaccine solution. Bath vaccination was another type of immersion vaccination based on uptake of antigens through gills, skin and the lateral line [24,25]. To-day the majority of the fish vaccines are delivered by injection, which is by far the most effective method compared to the oral and immersion route. The injection method is, however, labor intensive and not feasible for small or young fish. Future improvements of the oral and immersion delivery methods will certainly solve these problems.

3. Disease prevention

In a population of wild fish, diseases have been considered as a part of a normal biological process. In cultures of fish this situation gradually changed. The number of fish and the density increased. Diseases which were considered to be a phenomenon in the wild fish population, sometimes became a problem in a fish farm.

Disease prevention should be based on measures including three interacting factors, the agent, the host and the environment. However, aquaculture has some disadvantages compared to bio-production of terrestrial animals. Pathogenic microorganisms may be transmitted through water. Furthermore, disinfection is difficult, or, as in the marine environment, even impossible. Furthermore, the side effects and risks associated with chemotherapy reduced the use of antibiotics to therapeutic use and even that should be limited. Some environmental factors might be controlled, but others, like water temperature, could not be influenced. The resistance of the fish, based on natural or acquired immunity, was therefore a crucial factor for the health of farmed fish.

During the short history of modern aquaculture it is accepted that disease prevention based on stimulation of the immune system has become an integrated part of the management of aquaculture operations. The history of fish vaccination consists of both

success and failures. Due to scientific efforts and rapid transfer of scientific progress into practical measures, vaccination has become an important part of the development of the global aquaculture industry.

4. Scientific production – reviews and conferences

The scientific production in fish vaccinology and related areas has been considerable. A search on “fish vaccination” in scientific data bases showed a total of approx. 10,000 papers. Most of them are published during the last 20 years. The total number of publications was slightly above 100 at the end of the eighties [26].

Fish vaccination has been the subject of many review articles e.g. Refs. [4,8,20,26–34]. Several international conferences on fish vaccinology and the proceedings from these have contributed to the progress [35–39]. Fish vaccination became also the subject of many dissertations before the turn of the century, e.g. Refs. [40–46]. In addition, there are numerous dissertations on related subjects like fish immunology, fish bacteriology and disease prevention in fish farming. Disease prevention in aquaculture by vaccines has also been reviewed in textbooks and book chapters e.g. Refs. [47–50].

5. Salmonids

Whereas research in fish immunology has included different fish species, studies on fish vaccinology was mainly restricted to salmonid fish. In a chapter about furunculosis the status in the late sixties is summarized [51]. Under the headlines *Prophylactic chemotherapy* the following statement is made: “Although the practice of feeding low levels of drugs to control enzootic furunculosis may be useful, it must be done with caution to avoid toxicity to the fishes and alertness to possible appearance of drug resistant strains of *A. salmonicida*”.

As to vaccination the authors concluded that oral immunization against furunculosis with antigens incorporated in the feed was the only feasible way of routine immunization. However, the authors indicated that the era of chemotherapy in aquaculture should come to an end and vaccination should be used for disease prevention.

An early presentation of vaccination against vibriosis was made by Hayashi et al. [52]. Based on immunization studies they concluded that injection of a concentrated vaccine might be a useful prophylactic way for control of vibriosis in rainbow trout. One year later Ross and Klontz showed that enteric redmouth disease (yersiniosis) could be prevented in fingerling rainbow trout fed pelleted food containing bacterial cells of the redmouth bacterium (*Yersinia ruckeri*) [53].

During the seventies immunoprophylaxis became recognized as method for prevention of infection caused by fish pathogenic species of *Vibrio* and *Yersinia* in aquaculture. The first product license for a fish vaccine was issued in 1976 when an enteric redmouth bacterin produced by Wildlife Vaccine Inc. was approved [54]. The effect on morbidity and mortality was documented both by scientific studies as well as by experience from commercial operations. Protection could be achieved by inactivated vaccines without adjuvants administered by injection or immersion [4,55].

In the eighties a new costly disease initially named “Hitra disease” appeared in salmonid aquaculture in Norway. There was some dispute about the etiology of the disease. It was soon concluded that the disease was an infectious disease caused by a new pathogenic bacterium, *Vibrio salmonicida* [56]. Since 1988 most of Atlantic salmon and rainbow trout in Norway have been vaccinated, initially via immersion, but nowadays via injection, against this disease which was given the name cold water vibriosis.

In salmonids the great challenge in disease prevention was furunculosis caused by *A. salmonicida*. Based on the positive experience with prevention of *Vibrio*-infections using immersion vaccines, there were great expectations for similar effects with a furunculosis vaccine. However, immersion of furunculosis bacterins was found to give insufficient protection. Injection of simple whole-cell culture bacterins stimulated a protective immunity, but the magnitude and duration were less than desired.

In vaccines for terrestrial animals adjuvants had been added to vaccines for decades. Al-hydrogel which was used both in vaccines for man and animals, provided improved protection also when used in fish vaccines [41,42]. However, in order to achieve a long-lasting effect oil adjuvants, like mineral oils, were found to reduce morbidity and mortality to an acceptable level, both in challenge tests as well as in aquaculture operations [46]. The local reactions caused by adjuvants were recognized as serious side effects, but effective disease prevention was considered more important than animal welfare by authorities as well as by the industry and consumers.

Bacterins produced with antigens from *Vibrio anguillarum*, *V. salmonicida* and *A. salmonicida* and added mineral oil adjuvants contributed to effective control of diseases which without immunoprophylaxis would have caused great losses to the industry. The large amount of antimicrobials for disease treatment before introduction of vaccines was not acceptable for an industry which claimed to be sustainable. The impact of vaccination on the success of Norwegian aquaculture was expressed by a senior in the Norwegian aquaculture industry, professor Trygve Gjedrem, as follows: "The industry might have survived with the economic losses due to high mortality, but it could not survive with the negative effects of high use of antibiotics". Vaccination was consequently one of the factors contributing to the development of the salmonid aquaculture industry. The low figures for use of antibiotics in Norwegian aquaculture represent a documentation of a success story in the history of vaccinology [57].

The reasons for the success in fish vaccinology in Norway are many. Innovative scientists in public and private institutions and companies deserve to be acknowledged. However, good cooperation between the scientific community, authorities and the industry were also important contributing factors. Vaccines for fish diseases were developed, produced and tested experimentally in the field at a high speed.

Even the approval of vaccines by the authorities was a fast process in Norway with little bureaucracy. During early years of fish vaccination, licensing of fish vaccines was the responsibility of the Ministry of Agriculture. The regulatory requirements were few and approval of fish vaccines was considered to be a part of the learning process. Safety and efficacy in the field were emphasized. The transfer of responsibility to the Norwegian Medicines Agency in 1993 represented a change to a more comprehensive regulatory procedure in line with international rules. However, retrospectively it can be stated that the simplistic, but effective regulatory work during the first years of aquaculture in Norway contributed significantly to the decrease in antibiotic use in the industry without causing significant negative effects [58].

During the early years of aquaculture major viral diseases included infectious pancreatic necrosis, viral haemorrhagic septicaemia and infectious hematopoietic necrosis. For the latter two viral diseases, biosecurity has mainly been based on eradication of diseased populations, and research on vaccination was not prioritized. The first successful experiments on vaccination against these diseases included live vaccines, either avirulent or attenuated strains [59–61]. The live vaccines provided acceptable or even good protection under experimental conditions, but safety considerations stopped further work. Some of the vaccines showed residual

virulence to groups of vaccinated fish at a level which was unacceptable. The safety concern for other fish species in the aquatic environment also contributed to reduced research on vaccines which could be used in commercial operations.

Inactivated viral vaccines for fish have provided some effect, especially under experimental conditions. However, the protective immunity in the field by inactivated vaccines has been relatively low compared with the protection achieved by most of the bacterial vaccines. Consequently, the aquaculture industry has not been satisfied with the efficacy [62].

6. Non-salmonids

Much of the research in aquaculture was performed in cyprinid fish. That included also studies on immunization. In the 1970s Schäperclaus [63,64] used carp for studies of immune response and protection after vaccination with *Aeromonas* antigens using different administration methods.

The success with fish farming of salmonids stimulated aquaculture of marine fish species. In the Mediterranean countries production of sea bass, sea bream and turbot increased and so did disease-related problems. In USA bacterial diseases were a challenge for the channel catfish industry. Vaccines were introduced in many countries and continents for disease prevention [65]. Some of these were commercial vaccines with national or international license, some were autogenous or experimental vaccines used in a few fish farms.

Two important bacterial diseases for Mediterranean farmed species were vibriosis and photobacteriosis (pasteurellosis). Inactivated vaccines with the same antigenic composition as the microorganisms causing the diseases, were found to provide acceptable to good protection [66,67]. Vaccination against these diseases is now a part of the biosecurity program in many regions with production of sea bass, sea bream and turbot.

Bacterial diseases were the primary concern in the USA channel catfish industry. The primary bacterial pathogens in this industry are *Edwardsiella ictaluri*, *Flavobacterium columnare* and *Aeromonas hydrophila*. Vaccines have had limited use due to the extensive nature of the husbandry and non-satisfactory protection [68,69]. Current vaccines against infections caused by *E. ictaluri* and *F. columnare* providing good protection consist of attenuated live immersion vaccines [70].

Most of the global aquaculture is located in countries in Asia. So far vaccination has not become an integrated part of biosecurity in that region. However, there is a growing interest in disease prevention by vaccines in production of tilapia, pangasius and other species. The first commercial vaccine against a disease in pangasius was licensed in Vietnam in 2011 [71].

7. Success and failures

The history of fish vaccination is generally a story of success. The fact that a small fish can be protected against an infectious disease by immersion for a few seconds in a diluted vaccine solution is one example of the remarkable ability of living organisms to cope with biological challenges.

However, there have also been obstacles in use of the fish immune system for disease prevention. Studies of various fish species have showed that the basic mechanisms of immunity in fish, birds and mammals are similar. However, the history of fish vaccinology also includes results of studies showing differences between species with great influence on the strategy and methods for immunoprophylaxis.

Maternal immunity is fundamental part of protection against infectious diseases in newborn individuals of mammals and birds.

It would be reasonable to believe that a similar mechanism existed for fish. Maternal immunity has been found to be transferred from immunized mothers to the offspring in the ovoviparous guppy [72]. In salmonid fish the transfer of maternal antibodies seems to be at very low levels and insufficient to provide protection of the offspring against infections [73]. In a model study using zebrafish it was shown that maternal immunization caused a remarkable increase in complement factors C3 and Bf in the mother and offspring [74]. The embryos from immunized zebrafish were more resistant to the pathogen, *A. hydrophila*, than those from unimmunized mothers.

The failure of passive immunization is compensated by the early maturation of the immune system. Salmonid fish as small as 2 g were protected after immersion vaccination [75]. The duration of immunity and the development of immunological memory, both crucial in vaccinology, increased with size [76]. Maximum duration of protection was achieved in rainbow trout immersion vaccinated at a size of 4 g. In commercial aquaculture duration of protection needed is directly related to the length of the production cycle, i.e. the period between vaccination and harvest.

Effective prevention of several bacterial infections in fish by vaccination is well documented in the literature. However, infections caused by intracellular microorganisms are a challenge. This includes both viruses and intracellular bacteria. Attenuated vaccines and/or DNA vaccines seem to be a possible solution.

8. The pioneers

The history of fish vaccination includes the achievements by many scientists working in universities and research institutes. Their production is presented and documented in the scientific literature.

Vaccines are also commercial products, developed, produced and marketed by private companies. Manufacturers of fish vaccines were generally small businesses with a few enthusiastic pioneers involved in many parts of the process from ideas to use of the vaccine in the field. These industrial entrepreneurs were important for the progress in the early days of vaccinology. In addition to know-how about the product, they contributed with knowledge about correct use of the vaccines (Figs. 1–5).

Many of the early pioneers were excellent scientists who combined theoretical expertise and practical knowledge about disease prevention by using vaccines with a commercial interest. We



Fig. 2. Donald F. Amend.

realize, that a list of companies and pioneers can never be complete and fair for those involved.

The Colorado company, Wildlife Vaccines with Guy Tebbitt, John Rohovec and Thomas Goodrich (Fig. 1) as experts, was the first manufacturer with a licensed fish vaccine. The company produced bacterins for the domestic and international market. Tavolek Inc, a subsidiary of Johnson & Johnson, was the second company with a licensed fish vaccine. Keith Johnson and Donald F. Amend (Fig. 2) were technical experts involved in most parts of the development and production of the enteric redmouth vaccine and other bacterins. However, Tavolek Inc and Wildlife Vaccines operated at a time when the market was too small for a profitable business.

In the eighties Biomed Research Laboratories in Seattle entered the market. Stephen Newman, Tony Novotny, James Nelson and Robert Busch (Fig. 3) were a competent group of aquaculturists and vaccinologists with expertise from disease prevention in hatcheries in North-Western USA and for manufacturing of bacterins.

Two other small companies also deserve to be mentioned in the history of vaccinology. Aqua Health Inc. in Charlottetown, Canada



Fig. 1. Thomas Goodrich.

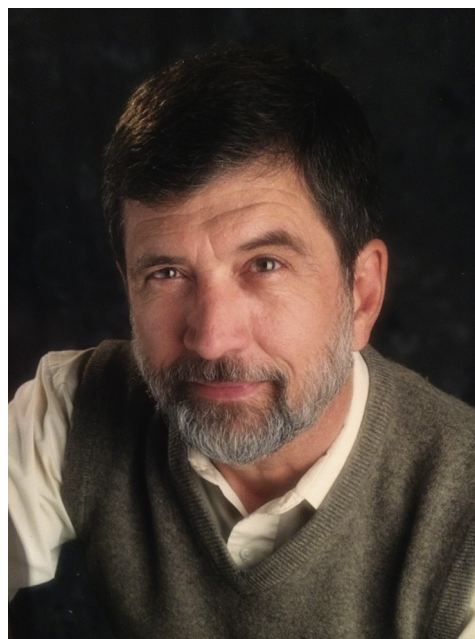


Fig. 3. Robert Busch.



Fig. 4. William D. Paterson.

with William D. Paterson (Fig 4) as technical and scientific expert and Aquaculture Vaccines Ltd. in UK, started as a subsidiary of Wildlife Vaccines, with Patrick D. Smith (Fig 5) as expert played a significant role in the early days of fish vaccination.

The rapid growth of the salmon production in Europe was the basis for the establishment of vaccine companies near the new emerging markets. Apothekernes Laboratorium, later Alpharma, was an international pharmaceutical company with headquarters in Oslo. The company saw a commercial potential in aquaculture and started fish vaccine production in Tromsø and later in Overhalla. In the beginning of the 1990s the company merged with Biomed Research Laboratories as part of the Alpharma formation. The aquaculture part of the company was later separated from the parent company under the name Pharmaq.

In Bergen, Norbio was established with the scientists from the university as experts. Norbio was sold to the Dutch company, Intervet, which later merged with Shering-Plough that had also absorbed Aquaculture Vaccines Ltd. The fish vaccine production has from 2011 been a part of Merck Sharp Dome.



Fig. 5. Patrick D. Smith.

Trading of companies is not unusual in the vaccine industry and some pharmaceutical companies and investors see the economic potential in fish vaccine production. The international pharmaceutical company Novartis has grown in the fish vaccine business, based on competence acquired with the Canadian company Aqua Health Inc.

In countries with a growing aquaculture industry vaccine companies have been established based on a possible economic potential. Companies like Centrovet in Chile and Kovax in Israel are examples of regional fish vaccine manufacturers with the goal to provide efficacious and safe vaccines for the aquaculture industry.

9. Concluding remarks

The history of fish vaccinology is a documentation of how the immune system of fish can be stimulated by vaccines to prevent accidental effects of pathogenic microorganisms. In a few years scientists in universities, research institutes and industry have produced basic and applied knowledge about the biology of microorganisms, the immune system of fish species in aquaculture, and this knowledge has been used for development, production, marketing and use of important products for the aquaculture industry.

Fish vaccinology has shown an amazing development in recent years. Most of the products are first generation vaccines, but a comprehensive scientific production and valuable practical experience are an excellent basis for development of improved products which will contribute to environmental, social and economical sustainability in global aquaculture.

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Review

Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review**M Rozas^{1,2} and R Enríquez³**¹ Faculty of Veterinary Sciences, Graduate School, Universidad Austral de Chile, Valdivia, Chile² Laboratory of Fish Pathology, Pathovet Ltd., Puerto Montt, Chile³ Laboratory of Aquatic Pathology and Biotechnology, Faculty of Veterinary Sciences, Animal Pathology Institute, Universidad Austral de Chile, Valdivia, Chile**Abstract**

The bacterium *Piscirickettsia salmonis* is the aetiological agent of piscirickettsiosis a severe disease that has caused major economic losses in the aquaculture industry since its appearance in 1989. Recent reports of *P. salmonis* or *P. salmonis*-like organisms in new fish hosts and geographical regions have increased interest in the bacterium. Because this gram-negative bacterium is still poorly understood, many relevant aspects of its life cycle, virulence and pathogenesis must be investigated before prophylactic procedures can be properly designed. The development of effective control strategies for the disease has been limited due to a lack of knowledge about the biology, intracellular growth, transmission and virulence of the organism. Piscirickettsiosis has been difficult to control; the failure of antibiotic treatment is common, and currently used vaccines show variable long-term efficacy. This review summarizes the biology and characteristics of the bacterium, including its virulence; the infective strategy of *P. salmonis* for survival and evasion of the host immune response; the host immune response to invasion by this pathogen; and newly described features of the pathology, pathogenesis, epidemiology and transmission. Current approaches to the

prevention of and treatment for piscirickettsiosis are discussed.

Keywords: control, epidemiology, pathogenesis, pathology, *Piscirickettsia salmonis*, piscirickettsiosis, transmission.

Introduction

Piscirickettsia salmonis was the first rickettsia-like bacterium to be known as a fish pathogen (Fryer *et al.* 1992). Since the first reports of piscirickettsiosis in Chile at the end of the 1980s, *Piscirickettsia*-like bacteria have been frequently recognized in various fish species farmed in fresh water and sea water and have significantly affected the productivity of aquaculture worldwide (Mauel & Miller 2002). The first record of a fish rickettsia-like organism (RLO) was described in fahaka pufferfish, *Tetraodon fahaka* (Hasselquist 1762), that originated from the Nile River in Egypt (Mohamed 1939).

A similar rickettsial septicaemia, 'parenthesis disease', has been recognized since 1970 in salmon held in sea water in British Columbia, Canada (Evelyn 1992). This septicaemia was first observed in 1970 and 1978 in pink salmon, *Oncorhynchus gorbusha* (Walbaum 1792), that were being cultured in seawater tanks for experimental purposes and was later observed in 1983 and 1984 in farmed coho, *Oncorhynchus kisutch* (Walbaum

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1792), and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum 1792) (Evelyn 1992). Subsequently, a similar rickettsial septicaemia was described in 1991 in Atlantic salmon, *Salmo salar* (Linnaeus 1758), farmed in sea water in British Columbia (Brocklebank *et al.* 1992). Gross pathology was consistent with the features of piscirickettsiosis described in Chile and was similar to pathological lesions previously noted in 1980 in coho and chinook salmon (Evelyn 1992). Later, a *Piscirickettsia*-like organism (PLO) was isolated from farmed Atlantic salmon from the eastern coast of Canada (Jones *et al.* 1998; Cusack, Groman & Jones 2002).

In addition, RLOs have also been recognized in Atlantic salmon farmed in Ireland and Scotland, United Kingdom. In Ireland, an RLO was observed in connection with low-mortality disease in Atlantic salmon post-smolts farmed along the western coast in 1991 (Rodger & Drinan 1993). No pathogen was isolated, but microorganisms similar to *P. salmonis* were observed in different tissues by microscopy. Between 1995 and 1996, four new outbreaks of the disease were described, and an RLO was isolated using the CHSE-214 (chinook salmon embryo) cell line and confirmed using anti-*P. salmonis* antibodies (Rodger & Drinan 1993). Finally, the association between the isolated agent and the disease was confirmed by experimental inoculation. In the same way, an RLO was isolated from seawater-farmed Atlantic salmon with high mortality in Scotland (Grant *et al.* 1996). The main histopathological lesion was encephalitis associated with vasculitis, and a great number of coccoid and basophilic microorganisms, approximately 1 µm in diameter, were found. The agent was isolated from brain tissue using the CHSE-214 cell line without antibiotics and showed a cytopathic effect (CPE) at 9 days post-inoculation at 15 °C; Koch's postulates were fulfilled. Further outbreaks of disease associated with *P. salmonis* in farmed Atlantic salmon have been described in Scotland (Birrell, Mitchell & Bruno 2003; Reid, Griffen & Birkbeck 2004), although the incidence and impact of piscirickettsiosis in Scotland are very low.

Between 1988 and 1992, an RLO was isolated from 51 Atlantic salmon farms along the western coast of Norway (Olsen *et al.* 1997). In total, 71% of these outbreaks occurred during the fall of 1988. Because of morphological and serological similarities to the type strain, the suggested name

for the organism was *P. salmonis* (Olsen *et al.* 1997). The main histopathological finding was hepatic necrosis and the presence of an RLO. The disease was frequently recorded after algal blooms, and the smolt pens tended to be overstocked with fish in poor condition (Olsen *et al.* 1997). Since then, the impact of infection caused by *P. salmonis* on the Norwegian industry has been low, perhaps because the better quality of smolt, better culture conditions and natural environmental conditions are not favourable for the bacterium or its possible vector (Olsen *et al.* 1997).

In Chile, the first outbreaks of piscirickettsiosis appeared by the end of 1989, although it has been suggested that the disease has been present in coho salmon since 1983 (Bravo & Campos 1989). Originally, piscirickettsiosis was described as affecting coho salmon (Bravo & Campos 1989; Cvitanich, Gárate & Smith 1990, 1991; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991), causing up to 90% of mortalities on certain farms (Cvitanich *et al.* 1990). Economic estimates conducted in 1989 determined losses of approximately US \$10 million, representing the mortality of approximately 1.5 million coho salmon (Cvitanich *et al.* 1990). Subsequently, piscirickettsiosis was also detected in Atlantic salmon and rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (Cvitanich *et al.* 1991; Cvitanich, Gárate & Smith 1995). The disease has been mainly described in sea water and brackish waters (Bravo & Campos 1989; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991) and, very occasionally, in fresh water (Bravo 1994; Gaggero, Castro & Sandino 1995).

In 2006, before the infectious salmon anaemia (ISA) crisis in Chile, the Technological Institute of Salmon (INTESAL) estimated that the direct economic losses caused by piscirickettsiosis during the on-growing phase in sea water were approximately US \$100 million and that losses of the potential concept of harvest were worth approximately US \$400 million (Cabezas 2006). These figures represented approximately 25% of total economic revenues from salmon exports during the same year. After the ISA crisis, piscirickettsiosis lost its key role in the health scene; today, however, together with the productive reactivation of the Chilean salmon industry, the disease has re-emerged as the main health challenge in the sector, thereby repositioning issues that are not yet

solved and are associated with diagnosis, treatment, prevention and control (Ibieta *et al.* 2011).

Currently, piscirickettsiosis is one of the most important threats to the sustainability of the Chilean salmon industry (Ibieta *et al.* 2011). Piscirickettsiosis has evolved over time; each new outbreak is increasingly insidious and refractory to treatments, and each has shown increased bacterial virulence, clinical and pathological severity and variable presentation under similar conditions of species, age and management measures (Leal & Woywood 2007; Marshall *et al.* 2007). In general, the salmon industry has focused its control strategy for the disease on antimicrobial therapies and vaccines. The use of antibiotics, both prophylactically and during early infection, may inhibit the growth of the pathogen, but such treatments have been largely unsuccessful in stopping disease outbreaks (Cabello 2006). Similarly, commercial vaccines against *P. salmonis* have not proven to be of high efficacy (Leal & Woywood 2007; Marshall *et al.* 2007).

A bacterium isolated from hatchery-reared juvenile white seabass, *Atractoscion nobilis* (Ayres 1860), in southern California, USA, was originally described as a PLO (Chen *et al.* 1994) and was identified as *P. salmonis* by the sequences of its small- and large-subunit ribosomal (16S and 23S) DNA and the internal-transcribed spacer (ITS) (WSB-98) (Arkush *et al.* 2005). The 16S rDNA homology with the type strain LF-89 was 99%. Additionally, the isolate WSB-98 of *P. salmonis* was recognized by the polymerase chain reaction (PCR) test designed by Mauel, Giovannoni and Fryer (1996). WSB-98 induced both mortality and disease similar to those of the piscirickettsiosis described in salmon in experimentally infected juvenile white seabass. In preliminary experiments, Arkush *et al.* (2005) also demonstrated that WSB-98 can be re-isolated from both chinook and coho salmon following cohabitation with experimentally infected white seabass in sea water at 16 °C.

An RLO in the brains of young seabass, *Dicentrarchus labrax* (Linnaeus 1758), cultured in the Mediterranean, near France (Comps, Raymond & Plassiart 1996), and an RLO from seabass farmed in Greece have been identified and described (Athanasopoulou *et al.* 2004). In addition, the DNA sequences of the 16S rDNA gene and the ITS region were compared with those published for *P. salmonis* strains, and it was found that the seabass PLO (SBPLO) was another strain of

P. salmonis that was closely related to the salmonid pathogens (McCarthy *et al.* 2005). Two ITS regions were observed in the seabass PLO, one of which contained tRNA genes.

An RLO was observed in farmed Atlantic salmon located in south-east Tasmania, Australia (Corbeil, Hyatt & Crane 2005). Whereas 16S rDNA sequence and phylogenetic analyses demonstrated that the Tasmanian RLO is related to exotic *P. salmonis* isolates, and particularly to the Chilean isolate EM-90, the Tasmanian RLO exhibits an ITS sequence divergence from other *P. salmonis* isolates. This divergence is principally due to a 19-bp deletion, which suggests genetic divergence from *P. salmonis*. In addition, the weak immunohistochemical staining obtained for Tasmanian RLO-infected tissues suggests antigenic similarity to the LF-89 isolate of *P. salmonis*.

Systemic infections in fish caused by gram-negative intracellular bacteria have been commonly referred to as either RLOs, due to morphological similarities with the true *Rickettsia*, or PLOs, following the description of *P. salmonis* (Fryer *et al.* 1992). Currently, many RLOs have been unequivocally shown to be *Francisella* spp., but it should not be assumed that all RLOs/PLOs that are not proven to be *P. salmonis* are in fact *Francisella* spp. (Colquhoun & Duodu 2011).

The agent

Taxonomy

Piscirickettsia salmonis was named to accommodate isolates from diseased salmon in Chile, of which LF-89 was studied in detail (Fryer *et al.* 1992), with 16S rRNA conforming to the gamma subdivision of the Proteobacteria, as for the genera *Coxiella* and *Francisella*. The bacteria of the genera *Neorickettsia*, *Rickettsia*, *Cowdria*, *Anaplasma* and *Ehrlichia* are members of the alpha subdivision of the Proteobacteria. Despite morphological similarities, the genera *Francisella* and *Piscirickettsia* belong to the Gammaproteobacteria and are therefore only distantly related to the true *Rickettsia* (Alphaproteobacteria; Mauel, Giovannoni & Fryer 1999). *Piscirickettsia salmonis* contains many genes that are absent from the *Francisella* genus and is thus likely to occupy distinct ecological niches (Sjödén *et al.* 2012).

The diversity within the 16S, ITS and 23S rDNAs reported by Mauel *et al.* (1999) is not

sufficient to split the genus *Piscirickettsia* into several species. More efforts are necessary to infer phylogenies by considering *P. salmonis* at the genome level, rather than only individual genes. Currently, the genus *Piscirickettsia* contains only *P. salmonis*, but Thomas and Faisal (2009) have described the apparent emergence of a novel *Piscirickettsia* species that causes disease in muskellunge, *Esox masquinongy* (Mitchill 1824), and yellow perch, *Perca flavescens* (Mitchill 1814). Phylogenetic analyses involving sequences of the 16S, ITS and 23S rDNA genes confirmed that muskellunge and yellow perch isolates were identical to each other, but not identical to *P. salmonis*, which devastates cultured salmonids, suggesting that the causative agent was likely a new species of *Piscirickettsia*.

Morphology and culture characteristics

Piscirickettsia salmonis is a gram-negative bacterium that is generally non-motile, aerobic, not encapsulated, pleomorphic, highly fastidious, usually coccoid and found in pairs or ring-shaped structures with an approximate diameter of 0.5–1.5 µm (Fryer *et al.* 1990, 1992). The bacterium replicates by binary fission within membrane-bound cytoplasmic vacuoles in the cells of susceptible fish hosts or fish cell lines. *In vitro* replication is optimal at 15–18 °C, is retarded above 20 °C and below 10 °C and does not occur above 25 °C (Fryer *et al.* 1990). Giemsa-stained tissue smears or imprints from infected organs exhibit darkly stained, pleomorphic *P. salmonis*, commonly in coccoid or ring form, inside cytoplasmic vacuoles surrounded by a membrane. The bacteria occur either singularly or in groups, giving the vacuole the appearance of a morula (Fryer *et al.* 1992). When *P. salmonis* is examined by electron microscopy, the bacterium displays the typical protoplasmic structure of a prokaryote and the cell wall of a gram-negative bacterium (Cvitanich *et al.* 1990; Fryer *et al.* 1990; Schäfer *et al.* 1990). *Piscirickettsia salmonis* has two surface membranes: an external undulated membrane and an internal cytoplasmic membrane (Fryer *et al.* 1990). The bacterium also has structures similar to ribosomes near the plasma membrane, fibrillar DNA in the central region and electrodense spherical structures (Fryer *et al.* 1990, 1992).

Piscirickettsia salmonis was previously considered to be cultivable only in eukaryotic cell lines (Fryer

et al. 1990; Lannan & Fryer 1991; Birkbeck *et al.* 2004a), but recent reports show that the bacterium may in fact be cultured on cysteine-enriched agar media, verifying the facultative intracellular nature of this fish pathogen (Mauel, Ware & Smith 2008; Mikalsen *et al.* 2008; Gómez, Henríquez & Marshall 2009; Yañez *et al.* 2012, 2013a). AUSTRAL-SRS broth allowed the production of bacteria with a 1.8 optical density when absorbance was measured at 600 nm after 6 days of incubation at 18 °C, and various passages did not alter the culture kinetics (Yañez *et al.* 2012). Recently, Yañez *et al.* (2013a) also described blood-free agar media (Austral-TSHem or Austral-TSFe) for use in the laboratory for the routine culture of *P. salmonis*. Artificial cell-free media provide a proper base to simplify the preparation of *P. salmonis* at a low cost for genetic and serological analyses, for the development of vaccines (Mauel *et al.* 2008; Mikalsen *et al.* 2008; Gómez *et al.* 2009; Yañez *et al.* 2012, 2013a) and for *in vitro* drug susceptibility testing (Yañez *et al.* 2013b).

A novel, genetically different, small infective variant of *P. salmonis* (sP.s) was characterized (Rojas *et al.* 2008) by analysing the sequences of the ITSs located between the genes encoding 16S rDNA and 23S rDNA. This sP.s variant was recovered from infected CHSE-214 cells and from naturally infected fish. The ITS region of the small variant is different from the ITS of the LF-89 strain from which the variant originates. Thus, the sP.s variant can be selectively amplified with a discriminatory set of PCR primers. Transcriptionally, sP.s is fully active and is specifically recognized by antibodies against the standard bacterium. Structurally, sP.s is smaller (<0.2 µm) than the size range of the standard bacterium (0.5–1.5 µm), and the variant's *in vitro* progeny appear to be identical to the progeny of the LF-89 strain. The sP.s is an infective variant of the reference strain and not a new strain, likely resulting from a survival strategy of the bacterium in response to limiting growth conditions. This variant may be responsible for horizontal infection in sea water (Rojas *et al.* 2008).

Genetic characteristics

A previous study based on an analysis of the rRNA operon of five isolates of *P. salmonis* (LF-89, ATL-4-91, NOR-92, SLGO-94 and EM-90)

suggested that sequencing data from three regions (16S-ITS-23S) provided similar phylogenetic information (>99.4% 16S rDNA, 99.1–99.7% ITS and 99.3–99.8% 23S rDNA similarities; Mauel *et al.* 1999). The five isolates under investigation were then found to be closely related to each other. However, the EM-90 Chilean isolate was described as unique, showing a slightly lower percentage sequence identity in its rRNA sequences (>98.5–98.9% 16S rDNA, 95.2–96.9% ITS and 97.6–98.5% 23S rDNA similarities). Nine of the base differences between EM-90 and the other isolates were found between bases 1003 and 1020. It was also found that *EcoRI* and *PstI* restriction sites located in the variable stem-loop region allowed the use of restriction fragment length polymorphism to differentiate EM-90 from the other strains (Mauel *et al.* 1999). The authors concluded that this isolate had diverged genetically from the others.

Piscirickettsia salmonis has two ITS regions: ITS A and ITS B (Casanova *et al.* 2001). Thus, more than one rRNA operon may exist. In both isolates, the smaller region (ITS B) corresponded to ITS sequences previously described for each isolate, and the larger region (ITS A) was nearly the same as the respective ITS B sequences, interrupted by an insert that contained two tRNA genes: tRNA-Ile and tRNA-Ala. It would be very interesting to determine whether the ITSs of *P. salmonis* isolated from other countries have similar characteristics. An ITS sequence analysis of 11 isolates of *P. salmonis* obtained from different salmon species and geographical regions in Chile demonstrated the existence of two different groups: ITSs with higher and lower electrophoretic mobility, including the LF-89 and EM-90 isolates, respectively (Casanova *et al.* 2003).

Only minor genetic variation has been observed between *P. salmonis* isolates from different salmonid host species or from diverse geographical locations (Mauel *et al.* 1999). A phylogenetic analysis of *P. salmonis* based on the sequences of the ITS and the 16S rRNA gene showed that the Scottish isolates conform to a genetic group, together with the Norwegian and Canadian isolates, whereas the Irish isolate conforms to a new group (Reid *et al.* 2004). Differences in the virulence of *P. salmonis* obtained from Chile (LF-89), Canada (ATL-4-91) and Norway (NOR-92) have been shown (House *et al.* 1999). Piscirickettsiosis was observed in fish injected with each of the three isolates, and for

each isolate, the cumulative mortality was directly related to the concentration of bacterial cells administered (House *et al.* 1999). The LF-89 isolate was the most virulent, with losses reaching 97% in the three replicates injected with 10^5 TCID₅₀, 91% in the replicates injected with 10^4 TCID₅₀ and 57% in the fish injected with 10^3 TCID₅₀. The ATL-4-91 isolate caused losses of 92% in the three replicates injected with 10^5 TCID₅₀, 76% in the fish injected with 10^4 TCID₅₀ and 32% in the fish injected with 10^3 TCID₅₀. The NOR-92 isolate was the least virulent, causing 41% mortality in the replicates injected with $10^{4.6}$ TCID₅₀.

Functional genomics

Analyses of gene content and genetic relationships may help to improve our understanding of the biology and evolution of *P. salmonis*. One concern is the comparative fluidity with which genes may be exchanged, such as by horizontal gene transfer, and the impact of this movement on the outcome of the taxonomic/phylogenetic process. The genomes of relatively few strains of *P. salmonis* have been sequenced. The complete genome of *P. salmonis* (LF-89 strain) was described in Chile (Valenzuela *et al.* 2001). The draft (95% of the genome) comprised an approximately 2 000 000-bp circular genome and a 1500-bp open reading frame (ORF), but currently, there are few whole-genome sequences available for *P. salmonis*. In addition, the genome sequencing data generated by Sjödin *et al.* (2012) represent a considerable advancement in our knowledge of the genome sequences of *Francisella* strains and the two distant but genetically related species *Fangia hongkongensis* and *P. salmonis*.

The presence of a toxin–antitoxin (TA) locus in the genome of *P. salmonis* has been described (Gómez *et al.* 2011). The *P. salmonis* TA locus, named Ps-Tox-Antox, includes its respective regulatory sequences. By *in silico* comparative genomics analysis of the Ps-Tox-Antox locus, the authors determined that this TA is homologous to the VapBC TA system of *Rickettsia felis* and to other chromosomal TA operons. Considering that the expression of the ps-Tox gene has been demonstrated to be highly toxic to *Escherichia coli* cells, the newly described module appears to be a potential innovative tool for pathogen control via peptide interference.

Piscirickettsia salmonis behaviour when exposed to stress conditions, which cause the bacterium to produce large cell aggregates that closely resemble typical biofilm structures, was described in a previous study (Marshall *et al.* 2012). The bacteria appeared to be embedded within a matrix that disappeared when exposed to cellulase, suggesting a polysaccharide composition that is typical of biofilm formation. Two lectins (ConA and WGA) showed a strong reaction with the structure, validating the exopolysaccharide composition of the matrix. The TA mazEF operon of *P. salmonis* exhibited induction of these genes at early stages of biofilm formation, suggesting that this formation might be an adaptive strategy for survival and persistence under stress conditions in marine environments.

On the other hand, the virulence factors of this pathogen are poorly known. *Piscirickettsia salmonis* secretes extracellular products (ECPs), and at least one of their components has cytotoxic effects *in vitro* and probably mediates some tissue damage *in vivo* in salmonid fish infected with this microorganism (Rojas *et al.* 2013). The almost complete inhibition of the *in vitro* effect of the *P. salmonis* ECPs by proteinase K treatment indicates their peptidic nature, and therefore, they can be categorized as exotoxins. In addition, several of these ECPs are thermolabile exotoxins that likely play a role in the pathogenesis of piscirickettsiosis (Rojas *et al.* 2013). *Francisella tularensis*, the most well-known species of this genus, is phylogenetically related to *P. salmonis*, there is no consensus opinion on the synthesis of exotoxins, and genes encoding toxins have not been found in this species. However, in *Francisella novicida*-like isolates, genes coding for putative RTX exotoxins have been recently found (Siddaramappa *et al.* 2011).

A novel and complete 863-bp insertion sequence in the *P. salmonis* genome has been recently characterized and named ISPsa2 (Marshall *et al.* 2011). This sequence has a novel 16/16 bp, perfectly inverted terminal repeat flanking a 726-bp ORF that encodes a putative transposase (Tnp-Psa). The putative transposase encoded within ISPsa2 (Tnp-Psa) carries conserved motifs that are also found in other transposases. The presence of a putative promoter region in frame with Tnp-Psa in ISPsa2 strongly suggests regulated self-expression for the IS and may represent a preliminary indication of the high genomic plasticity of this bacterial fish pathogen. *Piscirickettsia*

salmonis contains completely different sets of IS elements compared with *Francisella* (Sjödén *et al.* 2012), and the genome of *P. salmonis* is enriched with ISPsa1 and ISPsa2 (Marshall *et al.* 2011). These insertion sequences and, putatively, other mobile genetic elements in *P. salmonis* represent solid evidence that the adaptive potential of the bacterium resides in its versatile genome. In this context, the description of a TA locus in *P. salmonis* appears to be a natural consequence of this versatility (Gómez *et al.* 2011).

Evidence of the functional presence of four genes that are homologous to those described for Dot/Icm type IV secretion systems (SSTIVs) has been described for *P. salmonis* (Gómez *et al.* 2013). The Dot/Icm system, which is the major virulence mechanism of the related intracellular pathogens *Legionella pneumophila* and *Coxiella burnetii*, is responsible for these pathogens' intracellular survival and multiplication and may also apply to *P. salmonis*. The four *P. salmonis* dot/icm homologs (*dotB*, *dotA*, *icmK* and *icmE*) are expressed during both *in vitro* infection of cultured cells and growth in cell-free media, supporting the genes' putative constitutive expression. Additionally, as occurs for other bacterial systems, the acidification of cell-free media results in the overexpression of all four *P. salmonis* genes. Finally, researchers have also demonstrated that *P. salmonis*-containing vacuoles do not fuse with lysosomes, indicating that there is bacterium-driven interference in the endosomal maturation process that ensures bacterial survival, for which the Dot/Icm secretion system is responsible by delivering effector proteins within the host cell.

Although these works have provided some information about the virulence factors of *P. salmonis*, it is evident that further knowledge is required to fully understand the mechanisms of pathogenicity of this bacterium.

The disease

Clinical signs and gross pathology

A range of signs of infection may be present in salmonids infected with *P. salmonis* (Bravo & Campos 1989; Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Severely affected fish are dark in colour, show inappetence and lethargy and swim near the surface or edges of cages (Bravo & Campos 1989;

Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). The most consistent external signs observed during *P. salmonis* infections are pale gills resulting from significant anaemia, abdominal swelling and petechial and ecchymotic haemorrhages on the base of fins (Fig. 1a) and in the periocular and perianal zones. Infected fish often have skin lesions that range from small areas of raised scales to nodules (Fig. 1b) and shallow haemorrhagic ulcers (Fig. 1c, d; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Low levels of hematocrit indicate severe anaemia (Branson & Nieto Díaz-Muñoz 1991). However, during acute infections, mortality may occur without gross signs of disease.

Internally, *P. salmonis* infections spread systemically, resulting in serosanguinous ascites and swollen kidneys, livers and spleens (Fig. 2a). The most diagnostic lesions occur in the liver as subcapsular, gray-to-yellow mottled areas or as ring-shaped foci that are approximately 1 mm in diameter (Fig. 2b; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Fibrinous pseudomembranes on the heart and petechial haemorrhages in the visceral organs, swim bladder wall and skeletal muscle are often observed. In general, the intestine shows yellowish mucous content, and the stomach has transparent seromucous content (Almendras *et al.* 1997). *Piscirickettsia salmonis* was isolated

from the brain (Fig. 2c) of coho salmon farmed in Chile (Skarmeta *et al.* 2000), and it has been proposed that the erratic swimming behaviour observed in fish with piscirickettsiosis may be due to a pre-existing brain infection with more virulent isolates of the bacterium (Skarmeta *et al.* 2000). *Piscirickettsia salmonis* was also isolated from the brains of Atlantic salmon farmed in Scotland (Grant *et al.* 1996; Reid *et al.* 2004).

However, in recent years, the gross pathology of piscirickettsiosis has evolved towards the presentation of multiple diffuse skin ulcers all over the body. This pathology also includes opercula and peduncles, in addition to many caverns inside the skeletal muscle (Fig. 2d), regardless of the species of salmon and the geographical area of the sea water. The caverns inside the muscle can be observed as a clear area or with exudates. The pathological lesion is observed in both Atlantic salmon and rainbow trout; however, the lesion is more frequent in trout and is generally associated with the small variant of *P. salmonis*. Internally, classic pathological changes due to piscirickettsiosis, such as whitish nodules in the liver, renomegaly and splenomegaly, are not necessarily observed.

Histopathology

The most prominent microscopic lesions are found in the liver, kidney, spleen and intestine, but pathological changes in the brain, heart, skeletal muscle, ovary and gill can also be observed

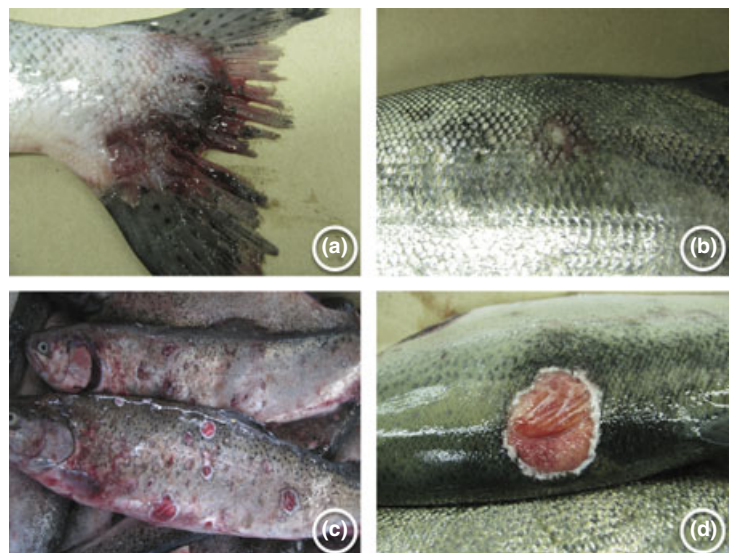


Figure 1 External signs associated with piscirickettsiosis in salmon species. (a) Petechial and ecchymotic haemorrhages on the base of fins. (b) Small areas of raised scales. (c) Multiple and diffuse shallow haemorrhagic skin ulcers in different areas of the body. (d) Focal skin ulcers and a loss of continuity.

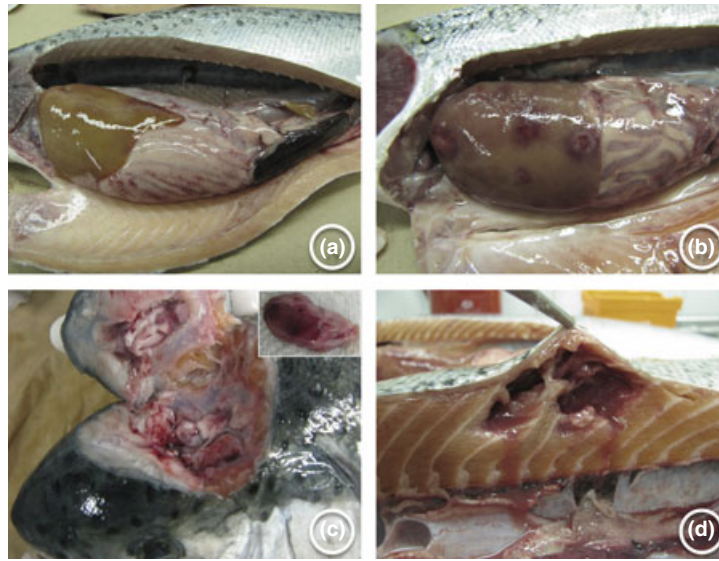


Figure 2 Gross pathology associated with piscirickettsiosis in salmon species. (a) Serosanguinous ascites, splenomegaly, pale liver and diffuse petechial haemorrhages are observed in the pyloric caeca and visceral fat. (b) Pale liver with subcapsular and circular gray-to-yellow mottled areas of approximately 1 mm in diameter and hepatic pseudomembranes. (c) Hyperaemia and diffuse haemorrhages in the meninges and petechiae in the encephalon. (d) Caverns with serosanguinous exudate inside the skeletal muscle.

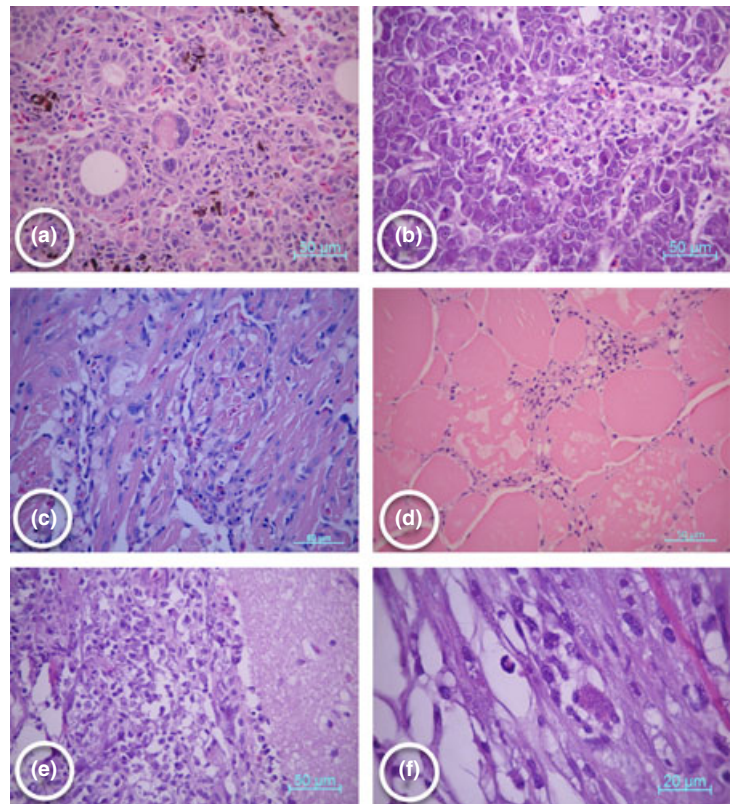
(Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Cvitanich *et al.* 1991). *Piscirickettsia salmonis* is commonly observed inside macrophages, within cytoplasmic or free vacuoles in the cytoplasm of the host cells. In certain fish, the bacteria are dark-coloured basophilic cells of approximately 0.5–1.5 µm, with a large nucleus and small cytoplasm, and are arranged in small groups inside the haematopoietic tissue (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991).

Multifocal necrosis of the haematopoietic cells in the parenchyma of the kidney is a feature of the more acute phase of piscirickettsiosis, which is followed by granulomatous inflammation (Fig. 3a). This necrosis results in a loss of haematopoietic cells that in turn leads to the observed anaemia that is characteristic of piscirickettsiosis. Glomerulonephritis with vacuolization and capsule oedema are also evident in the kidneys (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993). Vascular and perivascular necrosis and intravascular coagulation, resulting in fibrin thrombi within major vessels, are common findings in the liver (Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997). Multifocal necrosis of hepatocytes and diffuse infiltration of inflammatory cells are also observed in the liver (Fig. 3b; Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997). In more acute infections, the coalescence of areas of necrosis results in a more mottled appearance of the organ, rather than discrete

nodules. Within the areas of necrosis, macrophages can be found harbouring intracellular aggregates of *P. salmonis* (Fig. 3b). Focal areas of necrosis underlie the pale, circular lesions observed in more chronically infected fish (Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997).

Endocarditis, pericarditis and focal necrosis of the myocardium may also be observed, along with accompanying vascular changes similar to those in the liver and haematopoietic organs (Fig. 3c; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993; Olsen *et al.* 1997). Necrosis and diffuse chronic inflammation of the lamina propria and detachment of the mucosa are observed in the intestine. The pancreas, ovaries, mesentery, testicles, eyes, skeletal muscle, pseudo-gills, nasal capsule and adipose tissue have also been reported to be involved in certain *P. salmonis* infections (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993). Epithelial multifocal hyperplasia results in fusion of the lamellae, and focal necrosis and fibrin thrombi within lamellar capillaries may also be observed in the gills (Schäfer *et al.* 1990). Degeneration and necrosis of myocytes, a loss of striation, slight-to-moderate diffuse focal myositis and infiltration by polymorphonuclear cells (PMNs) (Fig. 3d) are often observed in the skeletal muscle (Schäfer *et al.* 1990; Rodger & Drinan 1993). The meninges show moderate granulomatous inflammation and thrombosis (Grant *et al.*

Figure 3 Microscopic lesions associated with piscirickettsiosis infection. (a) Degeneration and focal coagulative necrosis of interstitial and haematopoietic cells in the anterior kidney and diffuse granulomatous interstitial nephritis. (b) Focal coagulative necrosis of hepatocytes, diffuse multifocal hepatitis and intracellular aggregates of bacterial cells inside hepatocytes and/or macrophages. (c) Degeneration and necrosis of cardiomyocytes, moderate-to-severe diffuse focal myocarditis and infiltration by PMNs. (d) Degeneration and necrosis of myocytes, a loss of striation, slight-to-moderate diffuse focal myositis and PMN infiltration. (e) Degeneration and focal necrosis and moderate-to-severe diffuse focal meningoencephalitis. (f) Diffuse fibrohistiocytic infiltration of the leptomeninges, with the presence of macrophages with intracytoplasmic basophilic corpuscles.



1996; Skarmeta *et al.* 2000; McCarthy *et al.* 2005) and severe haemorrhagic meningoencephalitis (Fig. 3e,f).

Diagnosis

A diagnosis of piscirickettsiosis is based on the presence of the characteristic external and internal signs and microscopic signs of the disease in a salmonid host, combined with a demonstration of the presence of *P. salmonis* by one of several procedures (Almendras & Fuentealba 1997; Fryer & Hedrick 2003). Smears or impressions of the kidney, the liver, the spleen or infected cell cultures on glass or plastic substrates can be fixed and then stained with Gram, Giemsa, acridine orange or methylene blue stain for the direct observation of *P. salmonis* within host cells (Fryer *et al.* 1990; Lannan & Fryer 1991). In Giemsa-stained sections, the intracellular bacteria will appear as darkly stained pleomorphic organisms occurring in coccoid or ring form, often within host cell cytoplasmic vacuoles and frequently in pairs, with a diameter of 0.5–1.5 µm.

Piscirickettsia salmonis can be grown in cultured cells (Fryer *et al.* 1990; Lannan & Fryer 1991)

and in bacteriological media (Mauel *et al.* 2008; Mikalsen *et al.* 2008; Gómez *et al.* 2009; Yañez *et al.* 2012, 2013a,b). The tissues of choice for isolation of the agent include the kidney, liver and blood during active infection (Lannan & Fryer 1991), but the brain also represents an important tissue for detecting *P. salmonis* (Skarmeta *et al.* 2000). However, following initial detection in stained tissue smears, cultured cells or bacteriological media, the identity of *P. salmonis* must be confirmed by serological methods, for example, immunofluorescence (IFAT) (Lannan, Ewing & Fryer 1991), an enzyme-linked immunosorbent assay (ELISA) (Aguayo *et al.* 2002), immunohistochemistry (Alday-Sanz *et al.* 1994) or a recently developed single-dilution filtration-assisted chemiluminometric immunoassay (SD FAL-ELISA) that can be applied to measure anti-*P. salmonis* IgM in individual or pooled serum and mucus samples (Wilda *et al.* 2012), or molecular techniques, such as PCR (Mauel *et al.* 1996; Marshall *et al.* 1998; Corbeil, McColl & Crane 2003).

Both nested (Mauel *et al.* 1996) and single-step (Marshall *et al.* 1998) PCR assays were developed during the 1990s to facilitate the detection and

characterization of *P. salmonis*. The targeted ITS region of the rRNA operon is more variable than the 16S region exploited in the nested PCR, therefore allowing finer discrimination in the description of new *P. salmonis* isolates (Marshall *et al.* 1998). A real-time PCR assay was developed, and this assay demonstrated higher sensitivity than did the nested PCR (Corbeil *et al.* 2003). The real-time PCR assay has the advantage of being faster and minimizes the risk of cross-contamination that is inherent to nested PCR. Additionally, the assay allows the quantification of bacteria in samples. A real-time PCR assay has also been implemented to detect bacteria in fixed paraffin sections (Karatas *et al.* 2008). Recently, a multiplex PCR-based protocol was designed for the simultaneous detection of *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *P. salmonis* (Tapia-Cammas *et al.* 2011). Finally, an *in situ* hybridization assay for *P. salmonis* using two pairs of primers (PS2S-PS2AS and PS2S-PS3AS) as specific DNA probes against the bacterium has been implemented (Venegas *et al.* 2004).

Pathogenesis

Progression of pathological changes

Microscopic lesions caused by *P. salmonis* during naturally acquired infections can be found in numerous organs and tissues, which is characteristic of a systemic or septicemic condition (Cvitanič *et al.* 1990, 1991; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Although the sequential development of naturally acquired infections has not been described, initial infections likely commence when the physical barriers of the skin and/or gills are breached by the bacterium (Almendras *et al.* 1997; Smith *et al.* 1999). Replication of the bacterium results in raised, discoloured areas of the skin that may then progress to shallow ulcers, as observed under field conditions and following experimental application of the bacterium onto the skin with filter-paper patches (Smith *et al.* 1999). The direct application of a bacterial suspension onto the gills also initiates local infections that spread via the blood and then via major vessels to the parenchyma of numerous organs (Almendras *et al.* 1997).

Serosal spread is more characteristic of the infections induced by intraperitoneal (IP)

injections of the bacterium (Almendras *et al.* 1997). In injected fish, the capsules of the major organs in the peritoneum are sites of replication prior to invasion of the parenchyma. In the later stages of infection, the internal and microscopic pathological changes observed in fish exposed via different routes become similar, most likely because septicemia eventually occurs, even in cases of serosal spread of the bacterium (Almendras *et al.* 1997). The septic nature of infections is demonstrated by the presence of infected macrophages, which are visible in blood smears from heavily infected fish (Branson & Nieto Díaz-Muñoz 1991; Almendras *et al.* 1997). There is no recent scientific literature concerning the pathogenesis of the disease in terms of the progression of pathological changes and the load of microorganisms in different challenged fish organs and tissues. However, the pathogenesis of *P. salmonis* in challenged Atlantic salmon via IP, oral or gill infection has been described, in accordance with the progression of microscopic lesions in the liver (Almendras *et al.* 2000).

Interaction with the fish immune system and molecular pathogenesis

Piscirickettsia salmonis is capable of infecting, surviving and replicating in and disseminating from macrophage/monocyte (RTS-11) cell lines derived from the rainbow trout spleen with a similar phenotype as uninfected control cells, without inducing CPE (Rojas *et al.* 2009). Infected macrophages stop proliferating, and a fraction detaches from the plate and transforms into cells similar to non-adherent monocytes with proliferative activity. These results suggest that the infection of salmonid innate immune cells without inducing the important response of cell death facilitates the persistence of the bacterium and its subsequent dissemination into other tissues, favouring evasion of the first line of defence against pathogens. For the first time, the apoptosis of rainbow trout macrophages infected by *P. salmonis* has been characterized using techniques based on morphological changes and cellular DNA fragmentation in the host (Rojas *et al.* 2010). The findings show that bacterial survival and evasion of the host immune response play an important role in the establishment of infection.

In recent years, microarrays have been used to examine immune and physiological responses and

developmental processes in Atlantic salmon (Rise *et al.* 2004; Tacchi *et al.* 2011). Experiments based on microarrays and validated through qPCR to identify genes that are differentially transcribed in response to infection with *P. salmonis* in the macrophages and haematopoietic kidney of Atlantic salmon have been performed (Rise *et al.* 2004). In the macrophages of infected fish, 71 transcripts registered with increased regulation and 31 transcripts registered with decreased regulation. In the haematopoietic kidney of infected fish, 30 transcripts with increased regulation and 39 transcripts with decreased regulation were observed (Rise *et al.* 2004). Ten antioxidant genes, including glutathione S-transferase, glutathione reductase, glutathione peroxidase and cytochrome b558 subunits α and β , showed increased expression in the macrophages of infected fish, but not in the haematopoietic kidney. Genes with decreased expression were those associated with the immune response in the infected haematopoietic kidney, but not in infected macrophages (Rise *et al.* 2004).

Recently, an infection model of *P. salmonis* was used to evaluate the differential transcriptomic responses using a microarray analysis of the anterior kidney, the liver and the skeletal muscle in post-smolts of Atlantic salmon at 48 h post-infection (pi) (Tacchi *et al.* 2011). The obtained results show how the fish respond to the infection and how the pathogen potentially modulates the host immune response. The infection caused a significant alteration of transcriptional activity in all evaluated tissues. In infected fish, 886, 207 and 153 transcripts were differentially expressed in the liver, anterior kidney and muscle, respectively.

Transcripts related to immune responses were modified in all three studied tissues (Tacchi *et al.* 2011). The head kidney had the greatest increase in expression of immune-related transcripts. Overall, the data indicate that *P. salmonis* affects the immune system of the host, activating the innate immune response in the head kidney, the muscle and the liver and potentially inducing inflammatory responses in the head kidney and an interferon-mediated response in the liver. However, this pathogen may compromise the adaptive immune response in infected fish as a mechanism to escape host defences (Rise *et al.* 2004; Tacchi *et al.* 2011).

Transcripts involved in G-protein signalling pathways were down-regulated in all three

examined tissues in the challenged fish, and in particular, the levels of regulators of G-protein signalling (RGS) were found to be decreased in all tissues (Tacchi *et al.* 2011). These results suggest that suppression of G-protein signalling may be part of the mechanism used by *P. salmonis* to evade host antimicrobial defences (Tacchi *et al.* 2011).

The host cell protects itself from oxidative damage by synthesizing the strong antioxidant glutathione. The expression of genes involved in the response to oxidative stress was found to be up-regulated in all examined tissues (Tacchi *et al.* 2011). NADPH oxidase exhibited increased expression in the liver, indicating that the fish were likely experiencing oxidative stress. Interestingly, glutathione S-transferase activity was down-regulated in the liver, which resulted in the up-regulation of catalase in the head kidney. Genes encoding heat-shock proteins (HSPs) were also induced following infection by the pathogen. It is well known that these proteins can induce cellular and humoral immune responses to infectious diseases. This family of proteins functions as co-chaperones in stimulating the ATP-dependent activity of HSP70, which is up-regulated in this tissue and protects cells during lipopolysaccharide (LPS) infection by up-regulating the expression of sphingosine kinase 1 (SK1). Together, these results suggest that *P. salmonis* infection may affect the host antioxidant system, eventually causing cell death and necrosis, as observed in several tissues of moribund fish infected by this bacterium (Almendras *et al.* 2000).

Following infection, there was an increase in the expression of genes related to protein metabolism in the liver, with genes involved in both protein synthesis and degradation being up-regulated (Tacchi *et al.* 2011). Genes involved in energy metabolism in the liver increased in expression in challenged fish, whereas the expression of genes related to gluconeogenesis markedly decreased. In the muscle, the expression of genes involved in both protein synthesis and protein degradation was found to be decreased in diseased fish. This result suggests that *P. salmonis* may cause decreased protein turnover in this tissue, reflecting a down-regulation of both anabolic and catabolic pathways in the muscle following infection.

During the complex interaction between a pathogen and its host organism, the induction or prevention of apoptosis may play a critical role in the

outcome of infection. *Piscirickettsia salmonis* may also inhibit apoptosis (Rojas *et al.* 2010) by down-regulating genes encoding proapoptotic proteins and by inducing cell proliferation-related genes in both the liver and the head kidney (Tacchi *et al.* 2011). This phenomenon is in agreement with the findings of Rise *et al.* (2004), who also found a decrease in the expression of apoptosis-related genes, whereas genes related to cell proliferation and the cell cycle were up-regulated. Modulation of the apoptotic response and the proliferation of the host cell may be a mechanism that *P. salmonis* evolved to ensure the maintenance of host cells as the site of infection.

Epidemiology and transmission

Environmental factors

Both temperature and salinity affect the survival of *P. salmonis* outside the host (Lannan & Fryer 1994). *Piscirickettsia salmonis* can survive for extended periods in sea water but is rapidly inactivated in fresh water (Lannan & Fryer 1994). The period of extracellular survival is greater at cooler temperatures (5 °C) and decreases as the temperature increases. In one study, under experimental conditions, *P. salmonis* survived in sea water for at least 21 days at 5–10 °C, 14 days at 15 °C and 7 days at 20 °C. The pathogen did not persist at temperatures above 25 °C (Lannan & Fryer 1994). The highest incidence of outbreaks is observed in the fall and spring, likely due to the water temperature (9 and 16 °C, respectively) (Bravo & Campos 1989; Cvitanich *et al.* 1990). Generally, outbreaks appear after a period of high variation in the environmental conditions of the water, such as fluctuating temperature or an increase in the concentration of non-toxic algae (Branson & Nieto Díaz-Muñoz 1991). In addition, it has been suggested that feeding to satiation, improper nutrition and/or stress could be predisposing factors (Garcías, Mendoza & Carvajal 2005). Piscirickettsiosis has often been described in sea water and estuarine waters but has been occasionally reported in rainbow trout farmed in Llanquihue Lake, which originated from eggs imported from the USA that were always maintained in fresh water (Bravo 1994), and in rainbow trout farmed in fresh water at Chiloé Island (Gaggero *et al.* 1995). In the second study, the gross pathology was similar to that

previously described in outbreaks in sea water, and the characteristics of the growth of the pathogen *in vitro* corresponded to the characteristics of *P. salmonis* (Gaggero *et al.* 1995). However, the reduced natural onset of piscirickettsiosis in fresh water suggests that the source could be the sea (Lannan & Fryer 1994). The rareness of outbreaks in fresh water may result from the instability of the bacterium in this environment (Lannan & Fryer 1994).

Horizontal transmission and experimental trials

Although there is still conjecture about the major mode of transmission of *P. salmonis* under natural conditions, direct horizontal transmission has been experimentally demonstrated in sea water and fresh water (Cvitanich *et al.* 1991; Almendras *et al.* 1997; Smith *et al.* 1999). The virulence of *P. salmonis* was evaluated via the IP injection of fish with three different isolates of *P. salmonis* (LF-89, SLGO-94 and SLGO-95; Smith *et al.* 1999). Exposed fish inoculated with the SLGO-95 strain showed a higher and earlier accumulated mortality rate than those fish inoculated with LF-89. Although all salmonid species farmed in Chile are susceptible to piscirickettsiosis, experiments have shown that coho salmon are more susceptible than rainbow trout (Smith *et al.* 1996a). A 50% lethal dose (DL₅₀) and a 50% infective dose (DI₅₀) of 10^{2.8} and 10^{1.9}, respectively, were described in coho salmon (Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991).

Despite the low *in vitro* persistence of *P. salmonis* in fresh water, horizontal transmission has been shown to be enhanced by direct contact among fish (Almendras *et al.* 1997) and by an increase in culture density (Larenas, Contreras & Smith 1997a). A higher accumulated mortality rate (24%) in salmon farmed at a higher density (20 kg m⁻³) and a higher water temperature (14 °C) has been described (Larenas *et al.* 1997a). The pathogen enters the host through the oral route, gills or skin (Smith *et al.* 1999). Although intact skin and gills can be penetrated by *P. salmonis*, there is an increased risk of infection following injury to these organs (Smith *et al.* 1999). The bacterium may be excreted in bile, faeces and urine from live fish, making coprophagy another viable route of infection (Salinas *et al.* 1997; Smith *et al.* 2004).

Fish with IP and gill infections show significantly higher mortality than fish challenged orally

(Almendras *et al.* 1997). In general, the most effective transmission route is via the skin, followed by the intestine (Almendras *et al.* 1997; Smith *et al.* 1999, 2004). These mechanisms have been observed in both sea water and fresh water, although the low survival of the bacterium in fresh water minimizes the probability of disease onset (Almendras *et al.* 1997; Smith *et al.* 1999). Infection is considered to occur primarily through horizontal transmission because the bacterium is capable of surviving in marine waters for extended periods (Lannan & Fryer 1994). However, in fresh water, unless the bacterium is protected within host cells or other biological material, rapid inactivation makes successful horizontal transmission unlikely (Lannan & Fryer 1994).

The incubation period for piscirickettsiosis depends on the bacterial isolate, the dose at which the isolate is administered to the host, the route of infection, environmental factors and host factors such as immune status, physiological status, species and age (Lannan & Fryer 1994). Death from piscirickettsiosis has been reported as early as 2 days following the IP inoculation of rainbow trout with *P. salmonis* (LF-89) (Smith *et al.* 1999). Other studies have reported deaths 8–29 days after a similar IP inoculation (Garcés *et al.* 1991; Smith *et al.* 1996a), and fish infected via the skin, gills or oral route died 10–14 days after first infection (Smith *et al.* 2004). Piscirickettsiosis-related mortalities have been noted in salmon as early as 2 weeks following their introduction into the areas of infected sea water in Chile (Cvitanich *et al.* 1990). Based on the above information, the incubation period for piscirickettsiosis under natural conditions has been estimated to be 10–14 days.

A real-time PCR technique was developed to detect and quantify *P. salmonis* in samples of sea water to estimate the rest period before new fish can be introduced into seawater farms (Olivares & Marshall 2010). Water samples were collected at 10-day intervals over a 50-day period at both the surface level and a depth of 5 m. The bacterial load decreased to zero at day 50, which indicates that a 50-day rest period after the removal of fish from cages appears to be appropriate before seeding a new stock of fish. Determining the minimal effective bacterial load in the water column of seawater farms may be an important procedure to complement health management for disease control.

Bath or cohabitation challenge models accurately represent natural exposure and provide predictable results for mortality (Strand & Midtlyng 2007). A standardized cohabitation challenge model for *P. salmonis* in Atlantic salmon has been described (Strand & Midtlyng 2007). The first study aimed to evaluate the loss of titres of cultured *P. salmonis* after freezing and showed that both fresh and frozen inocula induced mortality, with curves following a typical dose–response pattern (Strand & Midtlyng 2007). In both cases, the saline-injected control group started to die after 3–4 weeks, reaching 95% cumulative mortality after 6–7 weeks. The group receiving the highest dilution of the frozen material followed the mortality pattern of the saline controls. Similar mortality patterns were observed in a parallel experiment with seawater-adapted smolts. A second trial aimed to investigate how the course of mortality was influenced by the ratio of inoculated (challenger) to non-inoculated (cohabitant) fish and showed that waterborne infection readily occurs in only 30% of challengers (Strand & Midtlyng 2007). The onset of the mortality of inoculated fish occurred on day 16, and cohabitant fish reached an accumulated mortality rate above 95% on days 34–35. A third trial aimed to investigate the temporal patterns of establishing waterborne infection using cohabitant fish and suggested that inoculation was ineffective in establishing lethal infection in all groups receiving diluted inoculum. However, waterborne infection was apparently established in all cohabitant groups at approximately day 21, and shedding may have been needed to reach a threshold before waterborne infection with *P. salmonis* occurred (Strand & Midtlyng 2007). In summary, cohabitant challenge can be achieved in both fresh water and sea water, with an incubation period of 3–5 weeks.

Reservoirs and vectors

The source and reservoir of *P. salmonis* in the natural environment and the bacterium's mode of transmission are unknown. There are many parasitic crustaceans in the marine environment that may serve as vectors for *P. salmonis* (Fryer *et al.* 1990), although no vector/reservoir has yet been identified (Olivares & Marshall 2010). One important role of a vector in the life cycle of rickettsiae is prevention of the desiccation of these fragile bacteria during transmission from host to

host. Because the bacterial cell is protected from desiccation in an aquatic environment, it is possible that no vector is required for *P. salmonis* and that fish-to-fish transmission may occur (Fryer *et al.* 1990).

Piscirickettsia salmonis can replicate in insect- and frog-derived cell lines, suggesting that the bacterium has the potential to persist in invertebrates and non-fish poikilotherms (Birkbeck *et al.* 2004a). The parasitic isopod *Ceratothoa gaudichaudii* (Milne Edwards 1840), commonly associated with farmed salmon in Chile, was identified as a host for *P. salmonis* using an indirect fluorescent antibody test (Garcés *et al.* 1994). *Piscirickettsia salmonis* may penetrate the skin without injury in the absence of vectors, and subcutaneous inoculation is capable of inducing high mortality (Smith *et al.* 2004), suggesting that ectoparasites may perform an important role in transmission [e.g. *Caligus rogercresseyi* Boxshall and Bravo (2000)].

Reservoirs of infection in marine finfish species have been suspected, but testing of non-salmonid species in Chile failed to demonstrate reservoirs of infection in non-salmonid finfish (Garcés *et al.* 1994; Olivares & Marshall 2010). However, the presence of *P. salmonis* was detected by PCR in Patagonian blenny, *Eleginops maclovinus* (Cuvier 1830), in Huillínco Lake's surrounding salmon farms, which was likely associated with the lake's high salinity (Rozas *et al.* 2009). This finding suggests that a reservoir may exist, but its role in the disease's epidemiology has not been fully explained. In addition, infections with *P. salmonis* are not restricted to salmonid fish, as the bacterium can cause a disease similar to piscirickettsiosis in non-salmonid hosts (Arkush *et al.* 2005; McCarthy *et al.* 2005; Thomas & Faisal 2009).

Vertical transmission

Piscirickettsia salmonis has been described in the ovaries, coelomic fluid and testicles of naturally infected salmon (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Cvitanich *et al.* 1991). The infection of ovary tissue in brood stocks that were experimentally infected with *P. salmonis* was observed in the stroma, the cells of the theca externa, the follicular epithelium and the cytoplasmic vacuoles of oocytes at different developmental stages (Larenas *et al.* 1997b). In a sequential study of infection by optical microscopy, *P. salmonis* was observed from day 7 pi until day 20 pi,

which indicates that fish eggs are infected from an early developmental phase in the ovary tissue and that the tissue can generate gametes that are viable carriers of the bacteria (Larenas *et al.* 2003). Experimentally, *P. salmonis* was detected in a moderate amount in the eggs, coelomic fluid and seminal fluid in approximately 10% of fertilized eggs from male and/or female rainbow trout brood stocks that were inoculated intraperitoneally (Larenas *et al.* 2003). In another study, all groups of fertilized eggs from male and/or female brood stocks that were inoculated intraperitoneally with *P. salmonis* were capable of generating infected but viable sac fry (Larenas, Zamorano & Smith 2005). Infected fry did not show signs of the disease but were capable of excreting the agent through the faeces (Larenas *et al.* 2005). Piscirickettsial adhesion complex (PAC) was observed in *P. salmonis* by scanning electron microscopy; these extensions allow the bacterium to adhere to the fish egg wall and to enter after 5 min of contact (Larenas *et al.* 2003). Piscirickettsiosis is less frequently observed in salmonid fish during the fresh water stage of their life cycle than after entry into the marine environment. This feature suggests that vertical transmission may not be common for *P. salmonis* (Fryer & Hedrick 2003).

Prevention and control

The efficient control of and treatment for piscirickettsiosis have been difficult to achieve because there are no efficient commercial vaccines (Leal & Woywood 2007; Marshall *et al.* 2007; Tobar *et al.* 2011), and antibiotics (Cabello 2006) have a limited effect on the disease. Thus, the prophylactic control of infections depends on good management practices; periods of fallowing have been one approach to limit the spread of the pathogen (Olivares & Marshall 2010).

Vaccination

Piscirickettsia salmonis antigens that are potentially important for the development of vaccines have been identified based on their reaction with convalescent salmon sera and have been detected using polyclonal and monoclonal antibodies against salmon immunoglobulins (Kuzyk, Thorton & Kay 1996; Barnes *et al.* 1998). Four protein antigens and two carbohydrate antigens with relative molecular sizes of 65, 60, 54, 51, 16 and

approximately 11 kDa have been described (Kuzyk *et al.* 1996). The carbohydrate antigens of 11 and 16 kDa appear to be mainly core-region lipooligosaccharides with lesser amounts of LPS (Kuzyk *et al.* 1996). Eight protein antigens that are likely specific to *P. salmonis* (108, 95, 60, 56, 40, 36, 32 and 20 kDa) were also reported, of which only three had molecular weights similar to those previously described (Barnes *et al.* 1998). Thus, the main antigens are likely those with molecular weights of 56, 36 and 20 kDa.

Structurally, *P. salmonis* lipid A strongly resembles endotoxic enterobacterial lipid A (Vadovic, Fodorova & Toman 2007). The major *P. salmonis* lipid A species represents the hexaacyl form, resembling the classical lipid A found in the *Enterobacteriaceae* family (Vadovic *et al.* 2007; Vadovic, Ihnatko & Toman 2011). This fact might be one of the reasons for the high endotoxic potency of *P. salmonis* (Vadovic *et al.* 2007, 2011), which could explain the cause of the disseminated intravascular coagulation described in cultured coho salmon infected with *P. salmonis* (Cvitanich *et al.* 1991). It appears that the endotoxicity of LPS molecules is primarily determined by the number, nature and arrangement of acyl chains and phosphate groups on the lipid A part of the molecule (Vadovic *et al.* 2011). In addition, both the composition and the structure of major molecular species of *Rickettsia typhi* lipid A resemble those found for the classical forms of enterobacterial lipid A, which have high endotoxicity (Fodorova *et al.* 2005).

Recently, the carbohydrate backbone structure of LPS from *P. salmonis* has been described (Vinogradov, Frimmelova & Toman 2013). The presence of two consecutive residues of diacetylated pseudaminic acid (Pse 5,7 Ac, 5,7-diacetamido-3, 5, 7, 9-tetradeoxy-l-glycero-l-manno-non-2-ulonic acid) in the LPS appears to be unique among polysaccharides containing this acidic sugar. Similarly, the presence of 4-aminoarabinose (Ara4N, 4-amino-4-deoxy-l-arabinopyranose) on O-4 of the alpha-GlcN1P of the lipid A moiety is a unique feature of this LPS.

There is a lack of field information regarding the immune response and protection generated by vaccines available in Chile, but there are several results derived from experimental clinical trials. In trials with a non-concentrated bacterin, significant protection against natural challenges with *P. salmonis* was demonstrated (Smith *et al.* 1997),

whereas fish receiving adjuvant and concentrated bacterin in the same trial showed no protection or greater susceptibility to infection. Additionally, a vaccine composed of live *Arthrobacter davidanieli* has proven effective in reducing mortality from an experimental challenge of coho salmon with *P. salmonis* (Salonius *et al.* 2005). Under field conditions in Chile, use of the vaccine led to a significant reduction in piscirickettsiosis mortality in coho salmon for over 10 months following sea transfer (Salonius *et al.* 2005).

In other trials, an increased relative percentage survival (RPS) was observed when coho salmon were injected with whole-cell bacterins in oil and water adjuvants (Kuzyk *et al.* 2001). Improved protection was observed for a vaccine prepared from recombinant OspA lipoprotein (17 kDa), resulting in RPS values of up to 83% (Kuzyk *et al.* 2001). However, there is a need for further improvement, especially regarding the creation of multivalency, to ensure wider protection against emerging isolates (Wilhelm *et al.* 2006).

In one study, a Scottish isolate of *P. salmonis* (SCO-95A) isolated from the CHSE-214 cell line was inactivated by heat (100 °C for 30 min) and formalin (Birkbeck *et al.* 2004b). Post-smolt Atlantic salmon were intraperitoneally administered vaccines containing 10^9 heat-inactivated or 10^9 formalin-inactivated *P. salmonis* in adjuvant, with a control group receiving phosphate-buffered saline (PBS) in adjuvant. After an induction period of 194 days at 10 °C, the fish were challenged by injection with 0.1 mL of *P. salmonis* (2×10^4 TCID₅₀/fish) into the dorsal median sinus and maintained in sea water at 7.5 °C or fresh water at 16 °C. Mortality in the control group reached 81.8%, whereas the heat- and formalin-inactivated vaccines conferred significant protection against *P. salmonis*, with RPS values of 70.7% and 49.6%, respectively. The nature of the protective antigen is unknown but may consist of LPS or a heat-stable outer membrane protein. Fish that survived a dorsal median sinus challenge of *P. salmonis* or were cohabitants showed a strong immune response to the bacteria.

In Chile, expression library immunization technology was used to study the protection of the coho salmon *Oncorhynchus kisutch* against infection by *P. salmonis* (Miquel *et al.* 2003). Purified DNA from *P. salmonis* was sonicated, and the fragments were cloned into the expression vector pCMV-Bios. The plasmid DNA was isolated and

purified, and 20 µg was injected intramuscularly into 60 fish, followed by a second dose of 10 µg that was applied 40 days later. As a control, fish were injected with the same amount of DNA in the vector pCMV-Bios, without an insert. Sixty days post-injection, the vaccinated fish showed a significantly higher titre of specific antibodies against *P. salmonis* than did the control group. The vaccinated and control fish were challenged 60 days after the second dose of DNA with 2.5×10^7 *P. salmonis*, corresponding to 7.5 times the LD₅₀. At 30 days post-challenge, 100% mortality was observed for the control fish, whereas 20% of the vaccinated animals survived. All surviving fish exhibited a lower bacterial load in the kidney than did control fish.

It has been suggested that HSP10 and HSP16 of *P. salmonis* are highly immunogenic in salmon and are thus *bona fide* antigens for inclusion in an experimental vaccine to control piscirickettsiosis (Wilhelm *et al.* 2003). Subsequently, recombinant HSP60 and HSP70 from *P. salmonis* were shown to elicit a humoral response when injected intraperitoneally into Atlantic salmon and conferred protection on fish challenged with *P. salmonis* (Wilhelm *et al.* 2005). In addition, a strong humoral response was reported against membrane-bound transglycosylase B (MltB) and transferrin-binding protein B (TbpB) when these proteins were injected intraperitoneally into Atlantic salmon (Wilhelm *et al.* 2004).

Furthermore, a protective effect against piscirickettsiosis was described as being elicited by a mixture of recombinant proteins in fish (Wilhelm *et al.* 2006). Fifteen *P. salmonis* ORFs encoding HSPs and other surface-exposed antigens were isolated and expressed. Seven of the most promising antigens were formulated into three mixtures, containing HSP60, HSP70 and FlgG (V1); the amino-terminal part of the protein TbpB and the amino- and carboxy-terminal amino acids of the protein MltB (V2); or a recombinant carboxyl region of the proteins Omp27 and FlaA (V3). All three were injected into Atlantic salmon with an average weight of 18 g to test the mixtures' protective efficacy. V1 and V2 elicited a strong protective response in a challenge with the pathogen and achieved the highest level of protection, with RPS values of 95% and 84%, respectively. The humoral response of formulation V1 persisted for 8 months after vaccination (2800 degree-days).

A highly immunogenic protein, ChaPs, was cloned as a unique ORF encoding 545 amino

acids, with a 57.3 kDa molecular mass, and its immunological potential was determined using naturally infected fish serum (Marshall *et al.* 2007). Interestingly, a sequence analysis of ChaPs has demonstrated that the protein is an HSP, which is a type of molecule that has already been identified and exploited in recombinant vaccine development (Wilhelm *et al.* 2003).

At present, 33 injectable vaccines against piscirickettsiosis are commercially available in Chile (containing the *P. salmonis* fraction in monovalent and polyvalent formulations; Table 1) (Agricultural and Livestock Service, Servicio Agrícola y Ganadero, S.A.G 2013a). Four are subunit vaccines, and 29 are inactivated vaccines. In total, 31 vaccines are injectable, and two are oral. The number of injected vaccine doses for the control of piscirickettsiosis in Chile has increased ninefold between 2005 and 2010, but the vaccines show variable long-term efficacy (Leal & Woywood 2007; Marshall *et al.* 2007; Tobar *et al.* 2011). Regardless of their nature and administration route, the vaccines' field results have not been sufficiently documented and have shown relatively limited protection, particularly up to 1800 degree-days after injected primo-vaccination in fresh water.

In general, groups of vaccinated fish show lower accumulated mortality rates compared with unvaccinated fish until winter of each year, but the vaccine efficacy decreases significantly and mortality rates increase in both vaccinated and unvaccinated fish at the beginning of spring (Leal & Woywood 2007). All injectable vaccines are relatively effective at preventing the initial piscirickettsiosis outbreaks that occur after the transfer of fish from fresh water to sea water for the ongrowing stage (Tobar *et al.* 2011), but after this initial outbreak, the fish are susceptible to new, more aggressive piscirickettsiosis outbreaks that correlate with weakening of the specific immune response elicited by the first immunization event (Tobar *et al.* 2011). These outbreaks usually affect large fish and occur 10–12 months after transfer, resulting in greater economic losses. Although protecting those fish by means of an injectable revaccination is an interesting solution, the method is extremely difficult, mainly due to economic, practical and operational issues.

Oral immunization presents an attractive alternative to injectable vaccinations (Tobar *et al.* 2011). The vaccine, consisting of an antigen

Table 1 Vaccines with provisional registration for use in salmonids in Chile

Disease	Vaccine type	Presentation	Laboratory
Piscirickettsiosis	Inactivated	Injectable	Agrovet Ltda. FAV Veterquímica S.A. Recalcine S.A. Centrovet Ltda. Tecnovax Chile S.A. Centrovet Ltda.
Piscirickettsiosis-IPN	Inactivated Subunit	Oral	Centrovet Ltda.
	Inactivated	Injectable	Pfizer Chile S.A. Agrovet Ltda. Centrovet Ltda. FAV Novartis Chile S.A. Tecnovax Chile S.A. Pharmaq AS Chile Ltda. Centrovet Ltda.
Piscirickettsiosis-ISA	Subunit	Oral	Centrovet Ltda.
Piscirickettsiosis-IPN-ISA	Inactivated	Injectable	Agrovet Ltda. Centrovet Ltda.
Piscirickettsiosis-IPN-Vibriosis	Inactivated	Injectable	Centrovet Ltda. Tecnovax Chile S.A. FAV Novartis Chile S.A. Pharmaq AS Chile Ltda. Pfizer Chile S.A.
Piscirickettsiosis-IPN-Vibriosis-Furunculosis	Subunit	Injectable	Agrovet Ltda.
	Inactivated	Injectable	Centrovet Ltda. Pharmaq AS Chile Ltda.
Piscirickettsiosis-IPN-Vibriosis-ISA	Inactivated	Injectable	FAV Novartis Chile S.A.
Piscirickettsiosis-IPN-Vibriosis-Furunculosis-ISA	Inactivated	Injectable	Centrovet Ltda. Agrovet Ltda. FAV Novartis Chile S.A. Pharmaq AS Chile Ltda.

Source: Agricultural and Livestock Service (S.A.G), Chile.

solution of injectable vaccine incorporated into an adhesive vehicle and supplied with the daily feed ration, induces a specific immune response at the local and systemic levels. Specific anti-*P. salmonis* antibodies are detected as soon as 300 degree-days after vaccination. Oral vaccination was able to protect fish against *P. salmonis* challenge when administered either as a primary vaccination or as a booster for an injected vaccine. The results show that oral vaccination is an efficacious method for the prevention of piscirickettsiosis outbreaks in sea water during the ongrowing stage.

Despite all of these positive results for piscirickettsiosis vaccines in experimental trials and although more than 10 years have passed since the first vaccine against *P. salmonis* was launched in the Chilean market, most commercially available vaccines in Chile have no apparent effect on reducing mortalities under field conditions. Meanwhile, piscirickettsiosis remains a major health threat to salmon farming in Chile. In our

opinion, the variable efficacy could most likely be attributed to the interplay of several factors related to the pathogen (virulence factors, pathogenicity, infectious pressure), host (genetic resistance, smolt quality, immune system and immunity) and environment (water temperature, algal bloom), including husbandry management (biomass, stress, zoning). In addition, several recent studies have shown that *P. salmonis* affects the immune system of the host, activating the innate immune response and potentially inducing inflammatory responses in the head kidney and an interferon-mediated response in the liver. However, *P. salmonis* may compromise the adaptive immune response in infected fish as a mechanism to escape host defences (Rise *et al.* 2004; Tacchi *et al.* 2011). Therefore, the infective strategy of *P. salmonis* for intracellular survival (Rojas *et al.* 2009, 2010) evasion of the host immune response (Rise *et al.* 2004; Tacchi *et al.* 2011; Gómez *et al.* 2013) and the nature of the fish immune system would

activate the host immune response only for a short period of time. However, work contributing to a better understanding of immunological activity and bacterial factors involved in the disease is as yet limited.

In comparison, the tularaemia vaccine, consisting of *F. tularensis* live vaccine strain (LVS), which is phylogenetically related to *P. salmonis*, does not elicit complete protection against lethal challenge with a virulent-type A *Francisella* strain (Schmitt *et al.* 2012). One factor that may contribute to this poor performance is the limited stimulation of antigen-presenting cells (APCs; Schmitt *et al.* 2012). This possibility suggests that insufficient activation of dendritic cells and macrophages could contribute to the incomplete protection engendered by LVS. Schmitt *et al.* (2012) examined whether the interaction of genetically modified LVSs with human APCs correlated with the effectiveness of tularaemia vaccine candidates. Stimulation of APCs *in vitro* was improved by genetic modification of an LVS, but did not correlate with efficacy against challenge *in vivo* (Schmitt *et al.* 2012). Because IFN- γ is a critical mediator of protective immunity against tularaemia, a diminished IFN- γ response likely contributed to the lack of protection after vaccination (Schmitt *et al.* 2012). Several recent studies have shown that IL-17 is also required for control of *F. tularensis* growth and the generation of an effective Th1 response following pulmonary challenge (Schmitt *et al.* 2012). Similarly, several trial vaccines against francisellosis in cod, *Gadus morhua* (Linnaeus 1758), based on simple whole-cell-based preparations have been tested both in experimental challenges and in the field in Norway. None have yet awarded a significant or satisfactory degree of protection (Colquhoun & Duodu 2011). Recently, a defined live attenuated strain providing protection against *F. asiatica* in fish has been reported (Soto *et al.* 2011).

Human rickettsial infection induces long-term immunity, but killed rickettsial vaccines stimulate incomplete protection. Additionally, a live attenuated mutant stimulates strong immunity but reverts to virulence (Walker 2009). Experimental studies in an inbred mouse model of *Rickettsia conorii* infection demonstrated the greater importance of CD8⁺ T cells than of CD4⁺ T cells and the crucial role of cytotoxic CD8⁺ T cell activity in the clearance of infection (Walker, Olano & Feng 2001). Humoral immunity did not play an

important role in recovery from infection, as antibodies to OmpA and OmpB did not appear until after the animal was well (Feng *et al.* 2004). Among the challenges is the identification of appropriate CD4⁺ and CD8⁺ T cell antigens and a means to stimulate long-lasting immunity (Walker 2009). Regarding host defence against *Coxiella burnetii* infection, specific anti-Phase I Abs play an important role in protection against the development of clinical disease at an early stage after *C. burnetii* challenge, whereas the T cell-mediated Th1 immune response is critical for the clearance and complete elimination of the organisms at the late stage of the infection (Zhang *et al.* 2007).

Chemotherapy

Although *P. salmonis* is sensitive *in vitro* to many antibiotics commonly used to control other infectious diseases in fish (Fryer *et al.* 1990), infected salmonids respond poorly to this treatment, perhaps due to an insufficient concentration of antibiotics to kill the pathogen within the host cell (Almendras & Fuentealba 1997; Mauel & Miller 2002; Fryer & Hedrick 2003). Clarithromycin, chloramphenicol, erythromycin, gentamicin, oxytetracycline and sarafloxacin inhibit bacterial growth in cultured cells (Fryer *et al.* 1990). Additionally, four *P. salmonis* strains (LF-89, EM-90, SLGO-90 and SLGO-95) were used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of oxytetracycline, oxolinic acid, flumequine, chloramphenicol and gentamicin (Smith *et al.* 1996b). More recent strains of *P. salmonis* (SLGO-94 and SLGO-95) presented a higher resistance level than did older strains (LF-89 and EM-90). Antimicrobial resistance to cotrimoxazole, furazolidone and penicillin and sensitivity to oxolinic acid were also reported in *P. salmonis* isolated from northern Ireland (Rodger & Drinan 1993).

Important variations in the patterns of the *in vitro* antimicrobial sensitivity of *P. salmonis* isolated from different salmon species, types of aquatic environments and geographical areas between 2007 and 2008 (Mora 2010) showed a potential risk of generating antibacterial resistance. Resistance has caused variability in efficacy during field treatment, increasing the number of therapies and adjustments in dosage. These results may indicate the existence of other interesting variables, such as productive and environmental aspects, particularly

temperature, salinity and oxygen concentration. These aspects, in addition to being important stressing agents for the host, may affect the pharmacokinetic properties of antibiotics (Mora 2010).

Similarly, it is possible to identify a potential risk associated with the restricted pharmacologic alternatives for controlling the disease in Chile. As set forth in the Supreme Decree No. 139 from the *Regulation of Pharmaceutical Products Exclusively for Veterinary Use* in 1995, to enable the importation of a veterinary drug and for the drug to be elaborated and commercialized in Chile, the product must be registered with the SAG (S.A.G 2013b). The implementation of any antibacterial therapy in Chile must be supported by a veterinary medical prescription. Table 2 shows the dosage of every active compound according to the SAG register.

According to information compiled by the National Fisheries and Aquaculture Service (Servicio Nacional de Pesca y Acuicultura, Sernapesca) through the General Sanitary Program of Data Record and Laboratories' Information Disclosure during January and December 2012, *P. salmonis* was the most diagnosed pathogen during the on-growing phase in sea water, with a proportional rate of 54.39% of cases (1041/1916; Sernapesca 2013a). The main infectious cause of the mortality of salmonid species during the on-growing phase in Chile is piscirickettsiosis (Sernapesca 2013a). In total, 26.6% of mortality on Atlantic salmon on-growing-phase farms has been classified as having an infectious cause, of which 69.4% is attributed to piscirickettsiosis (Sernapesca 2013a). In coho salmon, 15.5% of mortality on seawater farms has been classified as having an infectious cause, of which 60.3% is attributed to piscirickettsiosis. In rainbow trout, 46.5% of mortality on

seawater farms has been classified as having an infectious cause, of which 94.6% is attributed to piscirickettsiosis. This situation could explain why 82% of the total antibiotics used in 2012 in Chile, and particularly florfenicol (62%) and oxytetracycline (37%) (Sernapesca 2013b), were administered to control piscirickettsiosis (Sernapesca 2013b).

The use of florfenicol increased from 36.8% in 2007 to 56.7% in 2008, 61.3% in 2009 (Sernapesca 2011a) and 52% in 2010 (Sernapesca 2011b). Of the total quantity of antibiotics used in 2010, 72% were administered to control piscirickettsiosis, similar to the percentage used between 2005 and 2009 (Sernapesca 2011b). However, a significant decrease in the use of oxolinic acid and flumequine has been noted in Chile (San Martín *et al.* 2010; Sernapesca 2011b), mainly due to approved restrictions in the industry that prohibit the use of quinolones in fish over 1 kg in lots destined for the USA. Among the antimicrobial agents commonly used in aquaculture, for example quinolones, several are classified by the World Health Organization (WHO) as critically important for use in humans and thus should not be used in food-producing animals (Heuer *et al.* 2009). Oxolinic acid has also been removed from fish medicine used in several other salmon-producing countries.

Of the total quantity of antibiotics used in 2007, 66% were administered to Atlantic salmon, a percentage that was significantly reduced in 2008 (to 56.6%), 2009 (to 32.6%) (Sernapesca 2011a) and 2010 (30%) (Sernapesca 2011b). However, the amount used in rainbow trout gradually increased from 22.2% in 2007 to 32.5% in 2008, 44.6% in 2009 (Sernapesca 2011a) and 52% in 2010 (Sernapesca 2011b), which was likely associated with the drop in cultured Atlantic

Table 2 General dosage of the main antibiotics registered in Chile for use in salmonid species (San Martín *et al.* 2010)

Active principle	Presentation and administration routes	Dose	Withdrawal period (degree-days)
Oxolinic acid	Oral suspension at 20%, 50% and 80%	10–30 mg kg ⁻¹ of biomass per day for 10–15 days	450
Amoxicillin	Oral suspension at 50%	80 mg kg ⁻¹ of biomass per day for 10 days	300
Erythromycin	Oral suspension at 50% and 80%	75–100 mg kg ⁻¹ of biomass per day for 10–21 days	500
Florfenicol	Oral suspension at 40% and 50%	10 mg kg ⁻¹ of biomass per day for 7–10 days	200–300
Flumequine	Oral suspension at 10%, 50% and 80%	10–30 mg kg ⁻¹ of biomass per day for 10–15 days	300
Oxytetracycline	Oral suspension at 40%, 50% and 80%	55–100 mg kg ⁻¹ of biomass per day for 10–15 days	600

salmon biomass after the ISA crisis (Sernapesca 2011b, 2013b). However, the use of this antibiotic has increased again with the increase in farmed salmon biomass (Sernapesca 2013b). Thus, the amount of antibiotic used in Atlantic salmon increased to 62% in 2012 (Sernapesca 2013b), and the amount used in rainbow trout increased to 30% in the same period (Sernapesca 2013b). The amount of antibiotic used in 2007 was 642 g pure drug/metric ton of produced salmon (Sernapesca 2011a). Later, as a result of the ISA crisis, the subsequent drop in cultured biomass and the low prevalence of diseases, the amount of antibiotic used decreased to 516 g pure drug/metric ton in 2008, 289 g in 2009, 307 g in 2010, 318 g in 2011 and 413 g in 2012 (Sernapesca 2013b; Table 3).

Thus far, prevention and control strategies for piscirickettsiosis have generated partial results in Chile. Consequently, Sernapesca recently established the Specific Program of Surveillance and Control of Piscirickettsiosis through Exempt Resolution No. 3174, dated 28 December 2012, which has been in force since its publication in the Official Publication on 8 January 2013 (Sernapesca 2013c). Objective of the programme is to decrease the impact of the disease through the early detection and follow-up of cases with the application of timely and gradual control measures. In addition, a monitoring and best practices programme has been established to enhance antibiotic use and to detect antibacterial resistance (San Martín *et al.* 2010).

Conclusions

Much progress has been made in advancing our knowledge of *P. salmonis* over the past 5 years, but

there remain certain areas that require further research and development. It is necessary to improve our understanding of genetic and taxonomic relationships within the *Piscirickettsia* genus according to fish species and/or different geographical zones, including the recognition of new species and subspecies. This approach is motivated by the increasing number of whole-genome sequences that are becoming available and by the idea thought that the evolutionary history of the whole genome of *P. salmonis* must be more reliable than the history of one gene, considering the descent of species.

Improved molecular tools for the specific detection and diagnosis of piscirickettsiosis have been developed, but there remain major gaps in our understanding of the epidemiology and pathogenesis of the disease. There are insufficient data on the mechanisms by which *P. salmonis* might spread in the environment. A search for potential vectors and reservoirs of *P. salmonis* would greatly enhance our understanding of the natural history and control of piscirickettsiosis. In addition, work using the cohabitant model of piscirickettsiosis should continue to validate the model's applicability to vaccine efficacy, genetic selection and functional genomics studies.

Surveillance and monitoring programmes for piscirickettsiosis are needed to support prevention and control measures. Surveillance activities are necessary to define the extent of infection, and information from these activities should be used to monitor the progress of ongoing disease response programmes. Tracing investigations are also crucial to support decisions about the most appropriate control measures.

It is likely that only with the development of efficacious *P. salmonis* vaccines and a customized vaccination strategy will significant control of piscirickettsiosis be achieved. Antibody responses rarely provide protection and may promote certain aspects of pathogenesis and intracellular survival. It will be necessary to identify virulence factors and differences between high- and low-virulence isolates using genomics and proteomics tools. Improved knowledge about the progression of infection in fish and about how *P. salmonis* evades the host immune system is essential to developing vaccines with long-term protection. As is the case for tularaemia vaccines, the insufficient activation of APCs may contribute to the incomplete protection engendered by *P. salmonis* vaccines. Among the challenges is the identification of appropriate

Table 3 Antimicrobial volume used in the Chilean salmon industry between 2005 and 2012 according to the volume of harvested salmon

Year	Annual salmon production (thousand/metric ton)*	Antimicrobial volume (metric ton)*	Grams of pure drug/metric ton
2005	614	239	389
2006	647	344	531
2007	601	386	642
2008	631	326	517
2009	474	184	288
2010	466	143	307
2011	648	207	318
2012	819	338	413

Source: *National Fisheries and Aquaculture Service, Sernapesca.

CD4+ and CD8+ T cell antigens and a means to stimulate long-lasting immunity. Thus, it is important to characterize the immunological responses following immunization and challenge based on the expression of immune-relevant genes and proteins. Alternatively, it would be an important advance to develop and evaluate a *P. salmonis* live vaccine with avirulent/attenuated strains. Finally, it would be very interesting to perform a clinical field trial based on standardized and accepted protocols and with adequate control groups.

Antibiotic treatment during piscirickettsiosis control programmes must be well coordinated and should only occur once surveillance activities have been completed. The antibiogram of field isolates is exceptionally important for the effective pharmacologic control of *P. salmonis*, but efforts are needed to standardize *in vitro* antimicrobial sensitivity tests and to expand our understanding of the genetic and molecular pathways of antimicrobial resistance in *P. salmonis*. These efforts should be focused on the improvement in management routines, regulatory control of the use of antimicrobial agents, the implementation of prudent use guidelines and monitoring of the use of antimicrobial agents and antimicrobial resistance. All of this information is necessary to establish effective control strategies for the disease.

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Authors' contributions

MR participated in the conception and design of the review, acquired the statistical data for descriptive analysis and wrote the article. RE critically reviewed the article for important intellectual content. All authors read and approved the final article.

Competing interests

The authors declare that they have no competing interests.

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