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 a 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.

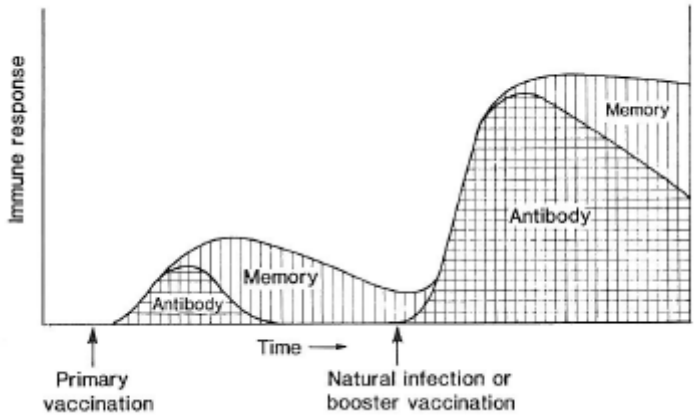


Figure 1. The immune response following primary vaccination and natural infection/booster vaccination [4].

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 feltvirus	+	
Pathogenicity for other species	+	
Contamination with other agens	++	
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
Ususally 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	

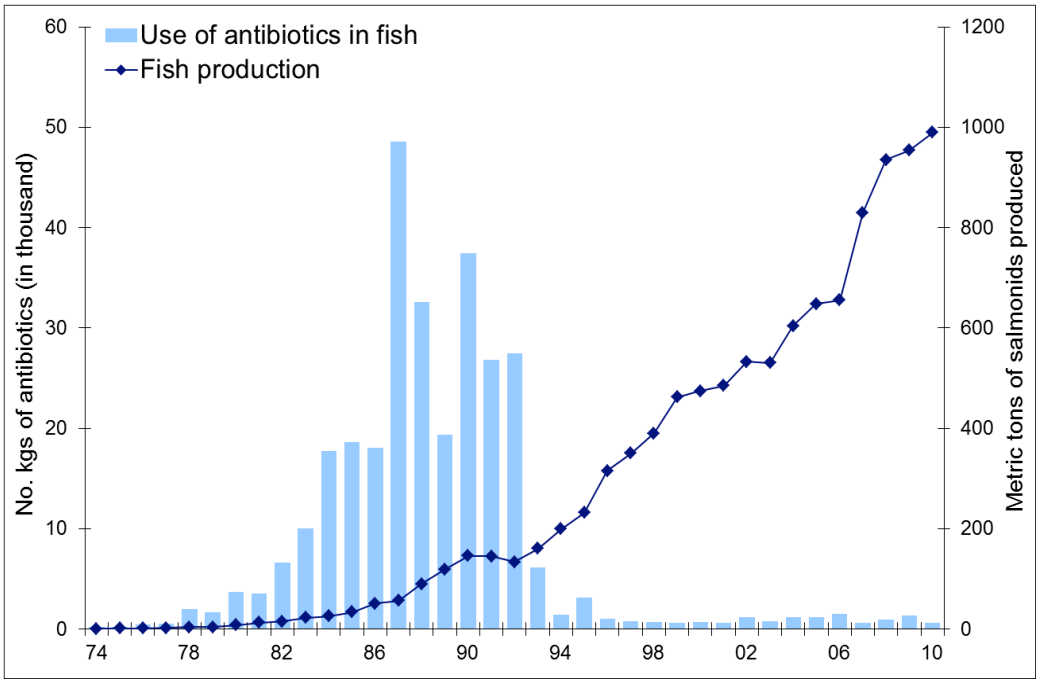
4. Vaccination of farmed fish

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 vibrosis. 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 Hitrasyke, 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 suceptible 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 melano-macrophages 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 (*tilvirkertillatelse*) 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 (*legemiddelmyndigheter*) 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
    - Outbreak of disease rarely occurs
    - 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
  - Two replicate cages
  - 1000 – 3000 fish per cage
  - 6 – 8 groups per cage
  - 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
  - 10 fish injected with a double dose per batch
- Potency
  - 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
  - One cage
  - 100 000 fish per cage
  - 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.

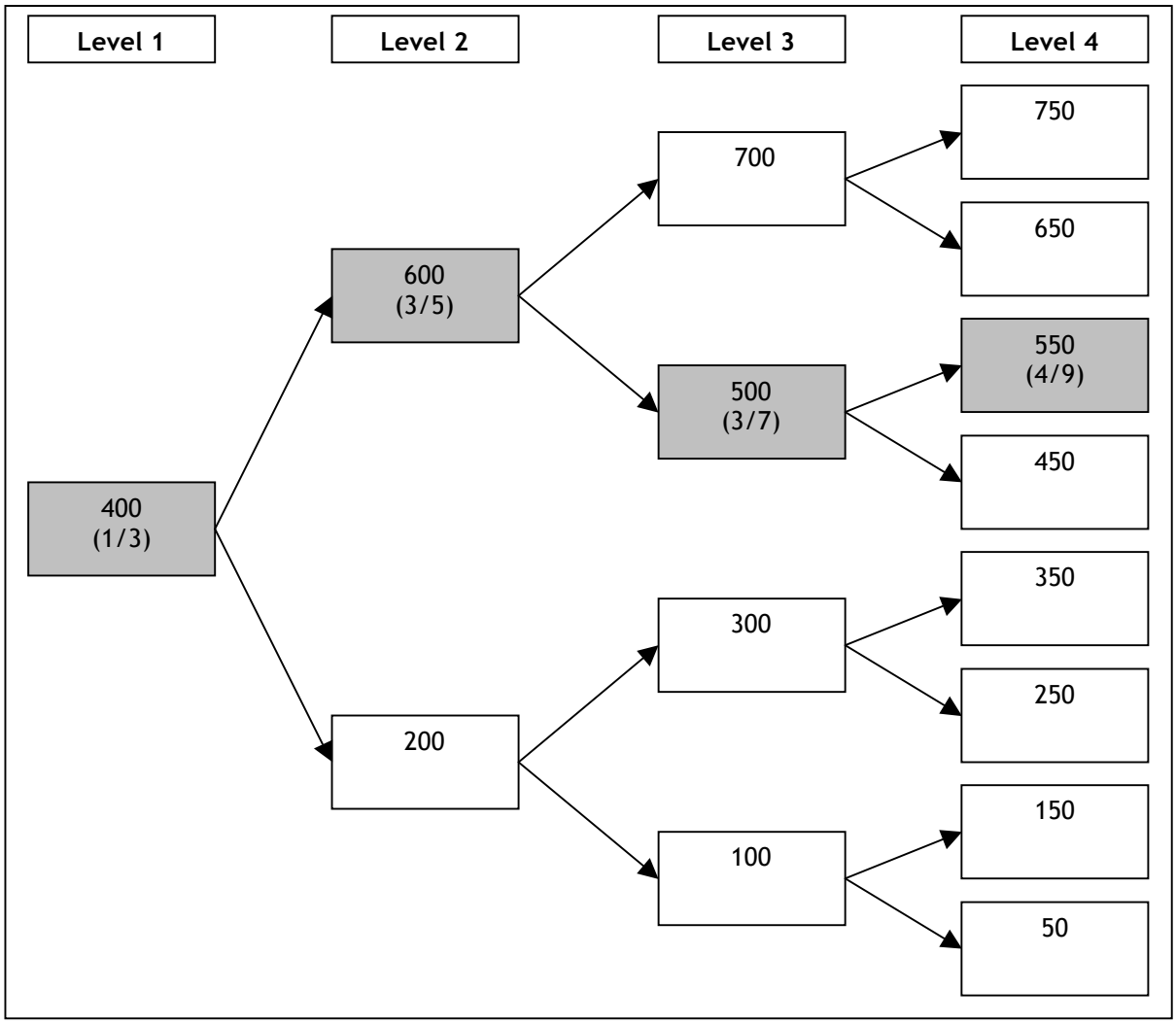
These findings indicate that an immunogenicity test can discriminate between vaccines of different antigen content. Following further validation this method therefore has the potential to replace challenge tests in vaccine tests.

6.2. Response Surface Pathway (RSP)

RSP was originally a technique developed in connection with LD_{50} 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:
$\mathbf{D_1 = (100 + 700)/2 = 400}$) [8]. To make the adjustment more sensitive in level 2, level two is calculated by: $\mathbf{D_2 = D_1 \pm (D_1/2)}$. 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: $\mathbf{D_3 = D_1 \pm (D_1/2) \pm (D_1/4)}$ and $\mathbf{D_4 = D_1 \pm (D_1/2) \pm (D_1/4) \pm (D_1/6)}$.



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 anitibody measurements.**

4. **Well-defined and trial specific humane endpoints in vaccine development and validation need to be developed and used.**

8. Acknowledgements

The authors wish to thank Anne Berit Romstad, Atle Lillehaug, Cecilie Mejdell, Liv Journ Reitan, and Kjetil Fyrand for helpful comments and discussions along the way.

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