Fish Vaccine Training Meeting for Assessors of Competent Authorities of the European Economic Area

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Proceedings of the meeting on Fish Vaccine Training Meeting for Assessors of Competent Authorities of the European Economic Area

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These proceedings are intended to assist colleagues in evaluating applications for marketing authorisation for fish vaccines. The proceedings have been compiled by the editors and reflect the presentations and discussions at the meeting of assessors in September 2005. Both the presentations given by the speakers and a summary of the presentation are included. The meeting was divided into two sessions which are also replicated in these proceedings.

Please note that the presentations may be opened using the hyperlinks in this and the accompanying screens. The presentations were given by the Speakers using PowerPoint and have been fixed as PDF images in these proceedings. For ease of use, it is recommended that the reader prints the accompanying summaries of each presentation. The summaries are accessible via the hyperlink on the top of this page. They are opened using an acrobat reader. Please scroll through the summaries in the document until you reach the presentation of interest.

The IMB gratefully acknowledges the valuable contributions of the speakers to the meeting.

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Chair of Session II: Una Moore

Welcome and Introduction
Pat O'Mahony,

Chief Executive, Irish Medicines Board, Dublin, Ireland
Welcoming delegates to the meeting, Mr. O'Mahony noted the importance of fish farming to the Irish economy. He acknowledged that he was honoured to host this training meeting in fulfilment of the commitment given in 2004 under the Irish Presidency of the European Union. He noted the high calibre of speakers and wished the meeting and its participants every success.

Fin Fish Farming: Significant Diseases and Trends:
Leo Foyle

Department of Veterinary Pathology, College of Natural Sciences, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland

The 'Blue revolution' is on its way! The speaker stated that capture fisheries and aquaculture have grown significantly over the last 30 years with global fisheries doubling its annual production since 1970 and aquaculture steadily increasing its production by approximately 10% per annum over the same time period. In the USA, aquaculture now exceeds the combined production of lamb, mutton and veal and by 2020 the Chinese authorities expect fish to become the country's main source of protein. China is the most important nation in aquaculture and has shown the largest growth (>70% in 2002). Traditionally, small local production was important in China but intensive multinational farms are now rapidly developing.

Currently, finfish dominate the aquaculture industry with a 50.4% share which is valued at 14.8 billion dollars. Of this share catfish, carps and cyprinids account for 16.7 million tonnes per year whilst salmon and trout account for the relatively small amount of 1.8 million tonnes.

There are two basic types of fish farm systems: freshwater and seawater. The latter can also be located on land. An example of a seawater salmon farm in Bergen, Norway that contains approximately two thirds of the total farmed population of fish in Ireland was shown. Often freshwater salmon farms incorporate artificial lighting regimes in order to speed up maturity.

The speaker declared that a significant factor with fish farms is the level of stress the fish can be under at times. These stresses arise from various sources such as nets and co-habitants, predators such as birds (cover and side nets are often employed as preventative measures), during transport where there can be a build up of metabolites like ammonia that can be detrimental to fish health. Then there is the risk of the spread of disease. Vaccination is important in the control of disease and disease spread, and of greater concern to some fish farmers than mortality is the main benefit of vaccination i.e. the improvement in food conversion ratio (FCR). This refers to the rate at which the fish convert meal to flesh.

In the past few decades, there has been a huge decrease in the use of antibiotics to control disease. In fact 2003 levels are as little as 0.5% of the amount used in 1987. Reasons for this include improved management and understanding of fish biology, and perhaps most significantly, due to the emergence of efficacious vaccines. These vaccines are now being used to greater effect to counter many of the major viral diseases of economic importance:

- Infectious Salmon Anaemia (ISA) – mostly found in salmon and is currently a List 1 notifiable disease
- Infectious Haematopoietic Necrosis (IHN) – the first licensed DNA vaccine to help prevent this condition in salmon

- Salmonid Alphavirus (SAV) – one of these, Salmon Pancreas Disease Virus causes necrosis of the acinar pancreas, skeletal and cardiac muscles and is probably under diagnosed outside Ireland. Responsible for a very high proportion (1:8) of deaths in farmed salmon that go to sea in Ireland. It is not notifiable making control difficult.
- Viral Haemorrhagic Septicaemia (VHS) – mainly affects rainbow trout but also turbot and was originally introduced to the USA and southern Europe from the Baltic Sea.
- Spring Viraemia of Carp (SVC) – common condition in the UK but not in Ireland. Experimental vaccines are currently being used.
- Infectious Pancreatic Necrosis (IPN) – affects numerous species in many parts of the world and is a commercially important disease. Controlling it is difficult.
- Viral Encephalopathy and Retinopathy (VER, VNN) – important in the Mediterranean, and in areas where halibut culture is common. Not significant in Ireland yet.

There are also various bacterial diseases of economic importance, many strains of which are country and species specific requiring the development of specific vaccines to suit individual fish species and individual industries:

- *Aeromonas salmonicida,* typical and atypical, the furunculosis family – the main bacterial disease in salmonid and some cod farming industries
- *Vibrio* spp. Multiple species affecting multiple fish species. The same fish species can be affected by a different *Vibrio* sp. depending on the country
- Piscirickettsia family – the major cause of disease in Chile, and of varying importance in other countries. A number of intracellular vaccines have been produced with little success to date.
- Bacterial Kidney Disease – antibiotics were used in the past for controlling the notifiable BKD but the particular pathogenesis of this bacterium is resulting in resistance becoming a problem

Another important disease is the protozoan disease, Proliferative Kidney Disease (PKD) which is of particular importance in trout farming. Vaccines are currently in development. There are also a number of other economically significant parasites affecting fish. Two such examples are *Lepeophtheirus salmonis* and the non-host specific *Caligus elongatus*.

Several diseases are currently emerging and increasing in prominence such as:

- Koi Herpes Virus – diagnosed in Ireland for the first time in summer 2005, the Asian strain of which has recently been found capable of affecting goldfish. An attenuated vaccine has been produced in Israel.
- Heart and skeletal muscle inflammation virus (HSMI) in Norway – the identity of which remains unknown at present
- Epizootic Haematopoietic Necrosis (EHN) – affecting Perch in Australia, but the related European Sheatfish and Catfish viruses, present in Europe also effect Rainbow trout and salmon

The speaker concluded by discussing some of the trends developing in the fish farming industry. One such trend is the emergence of novel species, such as the

seahorse and cod hatcheries in Carna, Co. Galway. Seahorses can be sold for up to €200-300 per fish. Aquaculture is trying to move away from the use of wild caught fish (meal) as the main component in commercial fish feed, and the area of feed technology, exploring and creating new sources of nutrition, is rapidly gaining importance. Finally, Blue water farming, exploring viable farms situated in the ocean, is becoming an increasingly popular topic.

Immunology of Fish
Dr. Mike Horne

Aquahealth, Scotland, UK

Fish immunology has not been studied to the same intensity as in mammalian species. Contrary to some reports, fish have well developed specific and non-specific immune systems although they possess less structurally defined components. Functionally the immune system in fish works as well as mammalians but structurally there are differences. Shrimp rely on non-specific immune mechanisms.

Innate immunity is non-specific (and not well studied) and is first line of defence against infectious agents. Physical barriers (skin, mucous, gut wall) contribute to this defence. Mucous contains anti-bacterial components but mucous consistency may change when fish are subject to stress. Gene silencing is also part of the innate immunity – small tRNA recognise external DNA. This was discovered in the 1990s. Tissue fluid contains various soluble factors, including growth inhibitors (transferrins), lysins, agglutinins and precipitins, enzyme inhibitors and interferon. Cells involved in the innate response include non-specific cytotoxic (NCC) cells and phagocytes. Melanocytes are well developed in fish – they envelop the pathogen (phagocytosis).

The adaptive immune system is composed of an anterior kidney (located under the backbone of the fish; this is also the site of haematopoiesis), the spleen (often enlarged in viral or bacterial infections), the oral cavity and intestine (however, unlike mammals, there are no Peyer's patches) and the thymus. There is no bone marrow or lymph nodes in fish. It is difficult to distinguish B and T cells in fish compared to mammals but they do exist. Free antibodies and memory B cells also exist (although it is not clear if the response is a prolonged primary response or true memory). There is only one class of antibody in fish however (IgM) but it performs most of the functions of many classes of antibodies of mammals. Antibodies are found in blood, gut lining and skin mucous.

The antibody response is classical – a low level titre following primary exposure (following an inductive period) with a secondary response with much higher titres following secondary exposure (after a very short period). Not all antibodies are protective. Moreover, circulatory antibodies against specific pathogen do not necessarily reflect the degree of protection. However, good protection can be achieved without detectable, specific antibodies. This is important as there is no protective correlation between antibody titre and challenge studies. Frequently, vaccinated rainbow trout do not show titres but are protected (local mucous immunity).

Fish are poikilothermic and therefore their immune response is temperature dependent. Different species have different permissive temperatures. Frequently, vaccination occurs at very low temperatures. Some fish show an immune response at temperatures as low as 1-2^{O}C to certain vaccines but not to IROMPs (iron regulated outer membrane proteins). All comparative data for fish vaccines should be on a degree day basis.

Cell mediated immunity is very important in fish. Good protection to viral vaccines given exogenously is not achieved possibly because of the poor stimulation of the MHC (major histocompatibility) class I pathway. Stimulation of the MHC class I pathway requires intracellular processing mechanisms. Good protection using bacterial vaccines can be achieved as the MHC Class II response is stimulated by exogenous pathogens (MHC class I response protects against endogenous pathogens whereas the MHC class II response protects against exogenous pathogens).

To date many immunomodulators have been identified in fish including the cytokines TNF-α and TGF-β, the interleukins IL-1, IL-6, IL-10 and the chemokines IL-8, MCP and RANTES. Many of these cytokines have similar functions in fish as those defined for other animal species. In the future it may be possible to use cytokine profiles produced in response to a specific antigen as a measure of efficacy of a vaccine thus reducing the need for animal testing.

Fish Vaccination
Marian McLoughlin

Aquatic Veterinary Services, Belfast, Northern Ireland.

Most of the current vaccines used in fish in Europe are for salmonids (salmon and trout mainly). The advent of fish vaccination (particularly oil adjuvanted vaccines) has had a very significant effect on reducing the use of antibiotics in salmonid aquaculture. Vaccination should be part of an overall health management package.

Salmon have a relatively long production cycle (circa 2 ½ years), comprising a fresh water phase (9-15 months) followed by a seawater phase (13-24 months). Vaccination is the best method to increase survival rate and profitability when used in combination with good nutrition, high-quality fish stock (fingerlings), good husbandry practices and good health management. Vaccination of young fish takes place between August and February. Fish are normally put to sea in the spring, although this cycle is changing to ensure the regular production of finished stock throughout the year.

Bacteria are responsible for approx. 55% of infectious disease in fish with viruses accounting for a further 23%. Globally important bacterial pathogens in salmon include *Listonella (Vibrio) anguillarum, Vibrio salmonicida, Moritella viscose, Aeromonas salmonicida, Aeromonas hydrophilia, Yersinia ruckeri, Renibacterium salmonis, Lactococcus/ Streptococcus sp* and *Piscirickettsia salmonis.* There are many fish virus pathogens in salmonid aquaculture including Infectious Pancreatic Necrosis (IPN), Salmon Pancreas Disease (SPD), Sleeping Disease of Trout (SD), Infectious Salmon Anaemia (ISA), Viral Nervous Necrosis (VNN) and Infectious Haematopoietic Necrosis (IHN).

The ideal vaccine will have sustained immunity and protection; be efficacious for a broad number of species, safe, well tolerated and capable of mass application. It will also be cheap, easily produced and stable and not interfere with diagnostic tests.

There are comparatively few licensed fish vaccines in the EU. The bulk of fish vaccines are adjuvanted inactivated (killed) viral or (primarily) bacterial antigens. Disadvantages of killed vaccines include the need for booster doses as such vaccines frequently produce a shorter duration of immunity compared to live vaccines, the need for adjuvants and the possibility of an increased incidence of adverse events. The advantages of killed vaccines include the fact that there is no risk of reversion of the pathogen to virulence and that there is a low potential for contamination of the vaccine during manufacture.

Modified live vaccines may theoretically be produced but are not used due to the risk of introducing disease into the aquatic environment. Such vaccines would provoke a good cell-mediated immunity and give a longer duration of immunity compared to killed vaccines. Disadvantages of live vaccines include the possibility of reversion to virulence and virus shedding, as well as some logistical issues as such vaccines are easily inactivated, have short shelf-lives after

reconstitution, require refrigeration and have a greater potential for undetectable contamination during manufacture.

Subunit vaccines include gene deleted viruses and viral vector vaccines. Gene deleted vaccines are produced following the isolation and removal of viral gene(s) that code for the 'non-required' proteins. This process is intended to decrease the virulence of the virus making it suitable for administration as a vaccine. Subunit vaccines are devoid of live organisms and therefore are very safe. They are relatively inexpensive to manufacture. However, the immunogenic response is restricted to selected antigens and the vaccine may not provide comprehensive protection against natural challenge in some animals.

Viral-vector vaccines are produced following the isolation of the specific gene that codes for immuno-protective proteins from a virulent virus and combining this gene with the DNA of a vector (non-virulent virus) which is then allowed to reproduce. The vaccine is comprised of the actual virus vector which 'carries' the DNA that, in turn, codes for immunoprotective proteins following administration to an animal. These vaccines are not capable of intracellular replication and therefore do not carry a risk of reversion to virulence. As the vaccine utilizes protective antigens which are very specific, the immune response may not be sufficiently broad in some animals to induce high levels of efficacy.

DNA vaccines involve the administration of genetic information directly into the host animal which then produces the antigens. DNA vaccines are most effective as they produce both cellular and humoral immunity. They may encode for several antigens or proteins and are therefore very efficient. They may be produced comparatively cheaply, they are easily stored and have a long shelf life. However, they cannot be used for certain microbes which have outer capsids which are made up of polysaccharides. Moreover, the public have misconceptions surrounding their use and much education is needed to dispel their fears.

The most commonly used antigens in fish to date are inactivated bacterial or viral vaccines. The route of administration used is usually by intraperitoneal vaccination. Most are given by hand although automation is increasing. Recombinant sub unit vaccines are used for IPN. DNA vaccines for IHN and VHS are in development. Such vaccines are given intramuscularly. Bath, dip and spray vaccination (immersion) are given to small fish. Oral vaccination would be desirable but is rarely available. Moreover, large quantities of antigen would be required and current vaccines tend to give weak protection of short duration.

The ideal site for intraperitoneal vaccination is on the pelvic spot just in front of the midline. Injection of fish is possible for fish of 15-20 g but ideally they should be 30 g or above. Most vaccination is done in fresh water. With the demise of malachite green there are problems of fungal infections post vaccination.

Farmers wish to have tailor made vaccines – they do not want to use antigens to protect against a disease which is not present on the farm, but this is not always commercially viable for the vaccine companies

Local injection site reactions are a problem for oil adjuvanted vaccines. Adhesions and melanisation with a local inflammation or peritionitis are expected. In relatively rare cases melanin and even multiple granulomata as a result of vaccination may occur. Factors which influence local reactions include the adjuvants, the antigens (bacterial are more aggressive than viral vaccines), the formulation, the dose volume (originally 0.2 ml dose was used but more recently 0.1 ml dose is used), the photo period (light manipulation influences growth and local reactions), temperature (higher temperature leads to greater number of reactions), size of fish (smaller the fish the greater the reaction), hygiene, and combination of all or any of the above.

The consequences of severe local reactions include reduced growth, increased feed conversion ratio, condemnations, slowing up the gutting process and animal welfare issues. Reactions are scored by the Speilberg or Midtlyng score (0 – 6). A score of 2 or less is seen as acceptable while a score of 4 or more is a matter for investigation. The distribution of scores is more important than the mean. It is necessary to examine a minimum of 30 fish per group and to examine at least 3 times during the field production cycles. There is also a melanisation score (score 0-3). The position and nature of adhesions and melanin deposits is important to note.

It is usual to compare the local reactions of a test product with that of a positive control as it is not generally possible to put fish to the sea without vaccination.

The ideal vaccination strategy in the future would involve a combination of immersion, injection and oral vaccines to ensure that all stages of fish production are protected from the relevant diseases. Currently the industry is relying on a single multivalent injection to provide this protection. Complimentary strategies include getting a better understanding of the fish immune system and using various in-feed immunostimulants to improve the fish immune response to both vaccination and disease challenge.

Discussion of the above presentations: Questions/Answers.
Regarding the use of immunostimulators, it appears that few controlled trials are available from which to judge their efficacy. However, the majority of feed-fish companies are putting such products into fish diets. Some of these diets are being sold commercially not only for fish at high risk periods but also for general feeding. B16 glucan is used as a feed additive. When given with antigens, there are literature publications to show that they have effects. They are really not able to replace vaccines but may be useful to support the efficacy of vaccines themselves. The stimulation of the immune system which they invoke is non specific. If glucans are put into very high quality diets, there may not be a demonstrable effect. Moreover, if given continually, they may damp down the immune response. With nucleotides, it was felt that there might be an enhancement of the speed of the immune response.

Control of Starting Materials used in Vaccine Production

Una Moore,

Department of Veterinary Medicine, Irish Medicines Board, Dublin, Ireland.

The speaker discussed the importance of using starting materials of biological origin which are free from extraneous agents in the manufacture of veterinary fish vaccines and gave details on how to minimise the risk of introducing extraneous agents into the finished product.

Contamination of a vaccine can constitute a serious safety risk to the recipient, the user and/or the environment. Freedom from extraneous agents is of paramount importance when the vaccine is being delivered directly to the fish. Fish vaccines are routinely administered by the intraperitioneal route, therefore, very low numbers of infectious particles may be sufficient to cause disease. Examples of biological products being released to the market place contaminated with extraneous agents were detailed, these included (a) in 2002, companion animal vaccines contaminated with feline parvovirus, (b) in 1999, a bovine respiratory vaccine contaminated with bovine viral diarrhoea and (c) in 1994, a canine vaccine contaminated with bluetongue virus.

The main source of contamination of vaccines with extraneous agents arises from the use of biological materials in the manufacturing process, although other sources of contamination may arise as a result of breaches of good manufacturing practises (GMP), operator error or cross contamination from one piece of equipment to another. However, as biological materials are considered the highest risk factor for the introduction of extraneous agents into the finished product, the remainder of the presentation focussed on how to minimise these risks.

Seed materials: To minimise the risk of introducing contamination from the use of seed materials, each seed used must be completely characterised. Bacterial seeds must be checked for purity, gram stain properties, biochemical profile (e.g. API sticks), morphology and identification (e.g. western blotting); there is no need to screen for viral contamination. For viral seeds it is necessary to ensure that all cell materials used for viral propagation are free from extraneous agents, the identity of the viral seed must be confirmed and its freedom from bacteria, fungi, mycoplasma and extraneous viruses assured. References to the European Pharmacopoeia (Ph. Eur.) on how to test for the presence of bacteria and fungi, mycoplasma and extraneous viruses were given as Monographs 2.6.1, 2.6.7 and 01/2005:0062, respectively. Master and working cell banks must also be fully characterised prior to use and subjected to the same characterisation studies as the viral seed.

In addition to the above, a number of specific screening tests must be conducted for seed materials used in fish vaccines; these are listed in the Note for Guidance ‘Specific requirements for the production and control of live and inactivated vaccines intended for fish’. Viral and cell seeds must be screened for the Viral Haemorrhagic Septicaemia Virus (VHSV) types I, II and III, Infectious

Haematopoietic Necrosis Virus (IHNV), Spring Viraemia of Carp Virus (SVCV) and Infectious Pancreatic Necrosis Virus (IPNV).

VHSV and IHNV are single stranded RNA enveloped viruses, members of the genus *Novirhabdovirus* and the family *Rhabdovirus*. Viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) are important economic diseases of salmonids and can result in high levels of mortality in young fish. VHS affects mainly rainbow trout in continental Europe. IHN affects predominantly farmed salmonids in western North America, Asia and Europe.

SVCV is a single stranded RNA enveloped virus, a member of the genus *Vesiculovirus* and the family *Rhabdovirus*. SVCV is an important disease of carp and can cause significant levels of mortality in carp of all ages. SVCV disease has been reported in most European countries where carp farming is practiced.

IPNV is a double stranded RNA non-enveloped virus, a member of the genus *Aquabirnavirus* and the family *Birnavirus*. Infectious Pancreatic Necrosis (IPN) is a highly contagious disease of young farmed salmonids and can induce mortality levels of up to 90% depending on the viral strain and age of fish. IPN has been reported in Europe, North and South America, Canada and Asia.

The Note for Guidance 'Specific requirements for the production and control of live and inactivated vaccines intended for fish' specifies that all seed materials must be screened for VHSV, IHNV, SVCV and IPN, however, advice regarding the appropriate methodologies is not provided. Knowledge of the correct screening methodologies is of vital importance as not all fish cell lines support the growth of these viruses. Use of inappropriate techniques may lead to erroneous results. Such advice is presented in the OIE manual of diagnostic tests for Aquatic Animals. Briefly,

- For detection of VHSV: The cell lines RTG-2 or BF-2 cultured at 15°C should be used as VHSV induces CPE under these conditions. Viral identity must be confirmed using IFAT , ELISA or RT-PCR.
- For detection of IHNV: The cell lines BF-2 or EPC cultured at 15°C should be used as under these conditions IHNV induces CPE. Viral identity must be confirmed using IFAT, ELISA, PCR or by a DNA probe.
- For detection of SVCV: The cell lines EPC or FHM cultured at 20°C should be used as under these conditions SVCV induces CPE. Viral identity should be confirmed using IFAT or ELISA.
- For detection of IPN: The cell lines BF-2, CHSE-214 or RTG-2 cultured at 15°C should be used as under these conditions IPN induces CPE. Viral identity should be confirmed using IFAT or ELISA.

Viral and cell seeds must also be screened for Myxosoma cerebralis (MC). MC is a parasite of salmonids and causes whirling disease. Severe mortalities of up to 90% can occur in young fish. The presence of whirling disease has been confirmed in the EU, New Zealand, South Africa and the US. Screening for MC is conducted by microscopic examination of samples stained with May-Grunwald.

Seed materials must also be screened for the presence of the bacteria *Yersinia ruckeri*, *Vibrio anguillarum* and *Aeromonas salmonicida*.

Yersinia ruckeri is the causative agent of the disease enteric redmouth which occurs in the EU, North America and Australia. Enteric Redmouth is usually seen in young rainbow trout farmed in fresh water but can also occur in Atlantic salmon in seawater. *Yersinia ruckeri* belongs to the family Enterobacteriaceae. *Yersinia ruckeri* is a gram negative bacteria and when cultured on standard bacteriological medium e.g. tryptone soya broth or blood agar at 18 - 22°C is characterised as whitish raised colonies with regular margins.
Vibrio anguillarum is the causative agent of the disease vibriosis, which is considered one of the most serious bacterial diseases of marine aquaculture. It has a worldwide geographical distribution. Vibriosis affects a variety of fish species including Atlantic salmon and Rainbow trout. *Vibrio anguillarum* is a gram-negative, motile rod shaped bacterium and requires sodium chloride for growth (halofilic). It can be cultured using tryptone soya broth supplemented with NaCl. Optimum growth temperatures range from approximately 13 - 17°C.

Aeromonas salmonicida subspecies *salmonicida* is the causative agent for the disease furunculosis, which is an important disease of wild and farmed salmonids throughout the world with the exception of South America. *Aeromonas salmonicida* subspecies *salmonicida* is a gram-negative, non-motile rod shaped bacterium. Typical strains produce a brown diffusible pigment in nutrient broth. Optimum growth temperatures range from approximately 18 - 22°C.

Seed materials, to which biological materials of ruminant origin have been added in their development, must undergo a risk assessment taking all the factors listed in the Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products. Only seed materials believed free from TSE can be used for vaccine production.

Substances of animal origin: Substances of animal origin are routinely used in the manufacture of fish vaccines. These materials can be ingredients of culture media e.g. bovine serum, trypsin, albumin or added constituents of vaccines or diluents e.g. mineral oil or gelatin. In order to reduce the risk of contamination from the use of these materials the following precautions should be taken:

- Only use materials of animal origin when necessary.
- Materials of animal origin must be sourced following the 'Strictest possible selection criteria (Ph. Eur. 01/2005/50205) i.e. (a) all materials must be sourced from healthy animals, (b) Animals should only be sourced from countries free from exotic diseases and (c) materials should only be sourced from animals from countries with a geographical BSE risk rating of I, II or III with justification.
- Prior to use materials of animal origin must be subjected to either a validated sterilisation/inactivation procedure or tested for the absence of extraneous agents (Ph. Eur. 01/2005:20205). Materials of biological origin must be screened in accordance with (a) the note for Guidance 'Specific requirements for the production and control of live and inactivated vaccines intended for fish', (b) the Guideline 'Table of extraneous agents to be tested for in relation to the general and species specific guidelines on the production and control of mammalian veterinary vaccines' and (c)

guideline on requirements and controls applied to bovine serum used in the production of immunological veterinary medicinal products.

Conclusion: In order to maintain product safety and minimise the risks associated with contamination of the finished product all materials of biological origin should be sourced using the 'strictest possible selection criteria', be extensively screened for the presence of extraneous agents and/or subjected to a validated inactivation/sterilisation procedure. All manufacturing processes should be conducted in accordance with GMP. Only then can the quality and safety of the vaccine be assured.

Issues Encountered in devising Laboratory and Field Efficacy Studies including Onset and During of Immunity

Lydia Brown

PHARMAQ Ltd, Norway.

The speaker outlined the regulatory framework governing licensing of vaccines which included the mandatory application of European Pharmacopoeia (Ph. Eur.) monographs as well as EU guidance documents and position papers which, although not mandatory, offered guidance on the production and control, the safety and the efficacy of vaccines. Relevant Ph. Eur. monographs in existence currently include the following:

- Evaluation of safety of veterinary vaccines (Ph.Eur. 5.2.6)
- Evaluation of efficacy of veterinary vaccines (Ph. Eur. 5.2.7)
- Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (Ph. Eur. 1521)
- Vibriosis (cold water) vaccine (inactivated) for salmonids (Ph. Eur. 1580) and
- Vibriosis vaccine (inactivated) for salmonids (Ph. Eur. 1581).

The speaker stated that the development of fish vaccines was a complex process which involved the production of three pilot batches, studies on safety, efficacy and duration of immunity, field testing in mini cages and in commercial scale conditions and finally batch testing of the product. All clinical studies must be scientifically valid, using predefined numbers of fish to obtain true differences between groups. This necessitated the use of proper statistical designs and methods. All test methods used in trials must be repeatable and reproducible. All clinical and mini cage studies should mimic the field situation in order to give a predicable response. Experience in Pharmaq has shown that more than 5000 fish are needed in the clinical development phase. Typically, this number would include the following:

- Virulence testing – 800 fish
- Development of challenge models – 800 fish
- Cross protection studies in the target species – 2000 fish
- Dose titration studies including challenge – 2000 fish.

Indeed, as many as 20,000 fish may be needed for the completion of development studies. Batch release of final product could necessitate studies on up to 15,000 fish while clinical commercial scale trials might involve several hundred thousand fish in a single study.

According to some Ph. Eur monographs, applicant companies may utilise antibody titres to determine efficacy if there is a good correlation between titres and laboratory efficacy trials. Some Ph. Eur monographs specify Relative Percent Survival (RPS) for vaccines. The speaker stated that, in her experience, the Ph. Eur method for determining efficacy of a vaccine is not always the best or most efficient method but that it is difficult to gain acceptance for changes to the monographs. Moreover, a specified RPS taken at a point in time may not be relevant in practice where this is delayed mortality in the vaccinated group.

The speaker noted that different strains of fish could have major effects on vaccine efficacy. Moreover, different sizes of fish or different year classes have different physiological classes and should not be compared. Many laboratory efficacy trials are conducted in freshwater, whereas the field challenge occurred in seawater.

The cage effects of treatment are very important. What goes on within a cage can overshadow differences between fish. Cohabitation models already used in Norway are promising. In order to ensure the proper conduct and interpretation of the trial it is necessary to mark the fish. Salmon have adipose tissue in the fin on their back. When the fish are anesthetised this fin may be clipped and serve as a marker. However, some disagree that it might affect the welfare of the fish. Dyes are also used to mark fish. However, as this is done in fresh water, when fish go sea, fish marked with a dye may not maintain their identification.

Concerning the practical difficulties of field efficacy trials, the speaker stated that 5-15 trials may be needed before a disease outbreak occurs which is severe enough to demonstrate the efficacy of the vaccine. Moreover, it is necessary to obtain the permission of the farmer for the conduct of the trial and to ensure their commitment for the duration of the trial. Sometimes a farmer might decide midway through the trials not to continue with the trial and harvest the fish. The speaker also stated that farmers often want all their fish vaccinated while animal test certificates often limit the number of fish tested. Furthermore, farmers sometimes forget about trial animals and harvest the fish without recording of the required data. Grading and sorting are also a problem – as currently there are few personnel available either in pharmaceutical companies or on fish farms to assist in these processes. It is also important that these field trials do not go on for too long as it becomes difficult to maintain the integrity of the study.

Field efficacy trials are often conducted in mini cages containing 1,000-3,000 fish per cage. However, field trials are not suitable for investigating and documenting the duration of immunity (especially for viral diseases) as there may not be an outbreak of disease on the farm and controlled conditions are difficult to maintain. Moreover, on occasions, the disease outbreak may be catastrophic with up to 40% mortality. There have also been documented cases of problems where fish are taken for sampling from farms and transported over long distances – such animals are likely to be stressed and succumb to disease. On farm duration of immunity studies have been replaced by studies conducted in the laboratory, albeit the relevance of the challenge investigations conducted in fish held for up to 12 in freshwater for anadromous fish is questioned.

Aspects of Experimental and Fish Trials with Fish

Paul J. Midtlyng

Centre for Veterinary Contract Research and Commercial Services, Ltd., Norway

The speaker emphasised that physiological processes in fish are principally temperature dependent (including embryonic development and growth) and stated that comparison of treatment groups is therefore only meaningful when fish are held under the same water termperature, or over the same (seasonal) period of time. This is also true of the immune response. Therefore, serosurveillance of fish has very limited value of fish sampled from cold waters (<10°C) as antibody response assays tend to yield outcomes near their 'limit of quantification'.

A further problem in planning experimental studies in fish is the 'unstandardised' nature of the animals – there are limited stocks of specific pathogen free stocks. Moreover, size differences develop rapidly under normal rearing conditions while external sex determination is usually not possible.

Local side-effects to vaccine emulsions conducted under too low water temperatures may not reveal the full harmful potential of the formulation. Published studies have shown that such reactions were higher in fish immunised in August-September (at water temperature >12°C) compared to fish vaccinated during winter (at temperatures <6°C). It was also noted that there is a limited repertoire of assay reagents available for fish immunology and that this situation is unlikely to change due to the limited size of the fish biomedical research market.

Genetically determined resistance to disease is another significant factor and such unknown genetic factors may interfere strongly with infection trials in fish. This may vary from 5%-95% of stock. Yearclass effects could also affect the outcome of challenge studies. In identically designed IPN virus challenge trials on immunised fish, considerable seasonal differences were found between treated and control fish – the level of mortality in control fish was found to be very low at certain times of the year rendering the trial invalid. For certain diseases, water borne challenge experiments, which mimic the field situation, are most relevant and studies with injection challenge may lead to misleading results.

The pen or 'herd' effect in fish trials is very important. Herd immunity effects can be of major importance in fish trials. Comparisons of vaccine efficacy between separate pens of (test 'x' and positive control 'y') vaccinated fish which show statistical differences between groups must be interpreted carefully as when the study was repeated using one cage containing subgroup of 15,000 fish vaccinated with vaccine x among 40,000 fish vaccinated with vaccine y, no statistical difference in response was observed.

In the discussion following the presentation, the speaker observed that concerning challenge models, with some viral diseases it is not possible to

reproduce mortality – other means of benchmarking efficacy are needed e.g histological scoring mechanisms or immunological markers.

Even controlled trials, which use adjuvanted formulations given intraperitoneally, may induce lesions of the peritoneal cavity which last for many months. In these cases intraperitoneal administration of the challenge organism may elicit non-specific immune mechanisms leading to false positive results.

A video on fish vaccination was shown. It was noted that fish must be starved for 48 hours before vaccination in order to avoid diseases caused by fish soiling the surfaces and contaminating the vaccination area.

Problems Encountered during the Registration of New Applications for Fish Vaccines

Céline Lorteau-Sourgen,

National Agency for Veterinary Medicinal Products, Food Safety Agency, France.

The speaker first outlined to delegates the documentation required for the assessment of a new application for fish vaccines.

The speaker then proceeded to outline the following points that should be taken into account when assessing registration files for fish vaccines. :

- The physiology of fish differs considerable to that of mammal and birds:
  - initial reduced size and slow growth. Frequently very small fish (weighing less than a few grams) have to be vaccinated which cannot be carried out on an individual basis. This is one of the reasons for developing particular methods of mass vaccination (bath / oral vaccination).
  - As fish are cold-blooded animals water temperature influences the kinetics of their immunological responses, their adverse reaction profile associated with vaccination and the clinical signs displayed after challenge i.e. the lower the temperature the longer the onset of immunity (after vaccination), of clinical signs (after challenge) and of adverse reactions (after vaccination). Therefore, particular attention must be paid to the conditions under which investigative trials are conducted. Trial conditions should reflect normal rearing conditions as closely as possible, with particular emphasis being on water temperatures.
  - Fish immune systems differs from mammals
- The environment conditions to which fish are exposed differ significantly to mammals and birds; these factors must be taken into account when devising investigative vaccine trials for fish.
  - Water quality: The quality of the water under which trials are conducted is very important as parameters such as oxygenation or bioburden may alter the health of the fish. Therefore, to avoid the introduction of bias in to the trial all environment parameters should be recorded and vaccinated and controls fish housed in the same tanks.
  - Route of administration of vaccine and challenge: The stress for fish associated with vaccination is greater than the stress induced in other species due to the handling (which may induce lesions) and the withdrawal of fish from the water. When assessing the adverse reaction to the vaccine it is often questioned as to what reference group should the vaccinated fish be compared with i.e. should a control group (not handled) or a placebo group (handled and mock vaccinated) be used? In the 1st case, the adverse effects of vaccination can be assessed; in the 2nd case, the adverse effects associated with the vaccine and vaccination can be assessed.
    - Injection: Administration by injection results in significant stress to the fish, it is labor-intensive and costly, but it is presently the most efficient route of vaccination. Lower quantities of antigen are needed compared to the other routes and an adjuvant can be used. Injection

vaccination is usually used in bigger fish or when other routes are not effective.

- Bath or immersion: When vaccination requires sequential immersion of fish in a concentrated bath, vaccine stability and efficacy in the last group of vaccinated fish must be assured. The vaccination unit is defined as the weight of fish to be vaccinated and not by fish numbers (1 kg i.e. 200 fish each weighing 5 g or 500 fish each weighing 2 g). This method is easy to perform and is suitable for the vaccination of small fish. However, it is less efficient than injection vaccination and exposes the environment to the vaccine.
- Oral route. The vaccine is a premix for medicated feeding stuff. This route leads to less stress but it is the least efficient route of vaccination. Attention should be paid to the stability of the vaccine, in particular when introduced into food and when exposed to stomach secretions. The interaction of the vaccine with the food is also, at least theoretically, a concern. The vaccine must be formulated in a way that the antigen is protected from destruction in the stomach yet capable of inducing an immunological response in the gut. It may be difficult to find feed producers favourably disposed to producing medicated feed because of the constraints associated with the production of medicated feedstuffs, as the demand for such a product is low. Exposure of the environment to the vaccine is also a concern when the vaccine is administered in this way.

- Handling of fish is an important stress factor and should be minimized where possible e.g.
  - the weight of the whole group or of a representative sample should be recorded instead of individual fish weights..
  - Daily observation is limited to recording mortality, food intake and behavior
  - At the end of the trial, particular attention must be paid to pathological findings (local reaction at the injection site, other lesions etc), as this is the only stage at which the fish can be individually and thoroughly examined.
- Environmental exposure to the vaccine: When vaccination is performed by either immersion or by the oral route, the environment will be exposed to the vaccine. For inactivated vaccines this is generally not of concern provided that the vaccine has been completely inactivated (i.e. inactivation procedure have been correctly validated) and the vaccine correctly controlled for the presence of extraneous agents, as theoretically this will prevent the release of live organisms to the environment. If live vaccines were to be administered by these routes, it would be necessary to carry out extensive assessments of the impact of the vaccine strain(s) on wild flora and fauna.

Based on the speakers experience of assessing applications for authorizations of inactivated fish vaccines (only inactivated bacterial vaccines have been registered in France to date [September 2005]) the speaker emphasized that the following information concerning efficacy must be borne in mind:

- Generally, virulent challenges are needed to assess vaccine efficacy (batch efficacy, onset, duration of immunity) as markers of protection have not yet been established
- The route by which the vaccine is administered and the fish challenged: it was observed that following intraperitoneal (IP) vaccination, fish were protected following an IP challenge however the level of protection was significantly lower when fish were challenged by the natural route. Efficacy against a natural challenge or using the natural route for a controlled challenge should be documented in registration files.
- The duration of observation after challenge should be sufficient to record delayed mortality in vaccinates as it has been observed that in some instances vaccination delays but does not prevent the onset of clinical signs.
- The strength of challenge should be sufficient to obtain clinical signs in controls but not too high to mask the efficacy of the vaccine.

Concerning the MUMS (Minor Use Minor Species) status, a definitive list of minor species has not yet established for immunological products; the IWP is currently working on the establishment of this list. Vaccines for fish may fall into the MUMs category, as the market for fish vaccines is limited.

There are no defined minimal information requirements established for fish vaccines, as the information required for each vaccine will depend on its intended use e.g. major or minor disease for the target species, major or minor commercial species, epidemiological situation etc. Bearing in mind that at present only bacterial inactivated vaccines are registered in France, the French Agency requires that (a) the quality of the vaccine has been demonstrated (paying particular attention to extraneous agent testing, validation of the inactivation, reliability of the batch potency test), (b) the safety of the vaccine has been demonstrated for vaccines indicated for salmonids and for other species that at least one safety study (laboratory of field) has been conducted, (c) the efficacy of the vaccine has been shown for salmonids but for other species the efficacy data could be limited to the batch potency test assessed by a challenge.

For all products authorized in France care is taken when devising the SPC to ensure that it reflects the information of the registration file and that the user is informed of the lack of the data, when applicable.

Advanced vaccine technologies
Mike Horne

Aquahealth, Scotland, U.K.

The speaker stated that fish is second only to poultry in the development of vaccines currently. In 2002 the number of doses commercially used in salmon reached 150 million in Chile, 125 million in Norway and 45 million in the UK. The main types of vaccines used presently in the aquaculture industry are (a) bacterins (killed bacteria) used in vaccines against *Vibrio* and *Aeromonas* (b) virins (inactivated virus) used in vaccines against IPNV, ISA (c) killed recombinants used in vaccines against IPNV, SRS, (d) live avirulent vaccines against *Edwardsiella* and (e) Live commensal – xenotype vaccines, used in vaccines against BKD. New technologies are being explored to aid in the development of new vaccines, some of which are currently in use and some of which are in development. These technologies include DNA vaccines, RNA vaccines, single chain antibodies, gene silencing and 'plantibodies'.

The speaker described DNA vaccines in greater detail. The basis for DNA vaccine technology is plasmid technology. A DNA plasmid is an independently replicating piece of circular DNA capable of producing many copies of a cloned gene. The plasmid is clearly defined and the sequence of each nucleotide is known. In addition to the DNA encoding the protein of interest the plasmid is made up of several different sequences cloned together, each of which has a defined function. These include an origin of replication, an antibiotic selection marker (such as ampicllin) to facilitate growth selection of the bacterial plasmid, a promoter sequence (CMV promoter commonly used) and a termination sequence.

One of the main advantages of plasmid technology is that the plasmid is incorporated into the cell and the protein is produced endogenously thus eliciting a better immune response (both humoral and cell mediated) compared to vaccines that present a protein exogenously.

Further advantages to DNA vaccine technology include:

- Very pure, totally defines
- No side effects from adjuvants or toxins
- No risk of infection or reversion
- Create a board immune response
- Antigen is identical to that in the pathogen
- Stable in transport and storage - thus cold chain is not a problem
- Rapid development path
- Maybe in the future will be cheaper than current vaccines.

The speaker then described the efficacy (as demonstrated by challenge studies) and stability of the Novaritis vaccine against IHN and also briefly discussed previous approved trials for DNA vaccines against West Nile disease in the American condor population and Leishmania syndrome in puppies. When preparing a DNA vaccine standard issues must be considered such as quality, safety and efficacy.

Recent data from mammalian models indicate that concerns regarding immune tolerance, autoimmunity or anti-DNA antibodies are not significant issues. An environmental impact study was completed and no significant issues were identified.

Bio-distribution, clearance and integration studies using IHN DNA vaccine were conducted using a quantitative PCR assay in order to 1) study migration of the plasmid vaccine following intramuscular vaccination, 2) determine the longevity of the plasmid in the major organs and 3) to prove plasmid does not integrate into the fish genome. Results obtained indicated that one day post vaccination plasmid DNA was detected in the liver, spleen, gonads and kidney. Three days post vaccination plasmid DNA was not detected in any of the tissues tested. Further studies indicated that plasmid DNA was detected at the injection site up to 700 days post vaccination thus indicating that plasmid DNA persists in the muscle at the site of injection. Environmental studies indicated that kanamycin resistance has not been transferred to control microflora. In order to examine the risks of self injection a single dose or a ten times overdose was administered to mice and no measurable effects were detected. In summary, safety studies indicated that tissue dissemination was restricted to the vaccination site within three days and that no evidence was observed of gene transfer in fish microflora, plasmid integration or adverse effects from accidental self injection. Studies on the vaccine clearance from the vaccination site are ongoing.

The speaker outlined the regulatory frame work for the assessment of DNA vaccines in Canada, US and the EU and remarked that DNA vaccines may come into Europe in due course and these will have to follow the centralised procedure. Single-Chain Antibody (ScAbs described as genes that produce antibodies) are created by a screening process that identifies an effective monoclonal antibody, a reverse process is employed to identify the gene of interest which is cloned in to a DNA plasmid which results in the production of an antibody fragment directly from the DNA plasmid. ScAbs technology is in development but is currently far from the market place.

Further developments in technology were introduced such as “plantibodies” which is the growth of vaccines and other pharmaceuticals in green plants. The main advantages of plantibodies are (a) very cheap source of specific protein (b) can be fed directly to animals and (c) are free of toxins. However the disadvantages are (a) 2 to 3 years time lag before sufficient yield for trial (b) modification of genes during development means a restart (c) resistance to GMOs and (e) concern about pollen spread. From a regulatory point of view the authorities will probably raise the questions (a) What is a batch? (b) Where is GMP relevant? and (c) how is consistent quality assured?

Another plant based technology in development is the use of *Chlamydomonas* as an expression vector. The advantages of this system are that it is cheap, *Chlamydomonas* produces natural adjuvanting properties from cell wall components and can be grown in fermentors under regulated GMP conditions.