Vaccines
Aquaculture

Identification of Petitioned Substance

Vaccine Names:

Cyprinid herpesvirus type 3 vaccine, modified live virus

Edwardsiella ictaluri Vaccine, Avirulent Live Culture

Aeromonas salmonicida bacterin

Aeromonas salmonicida-Vibrio anguillarum-ordalii bacterin 303A

Vibrio anguillarum-ordalii bacterin

Arthrobacter davidanieli vaccine, live culture

Infectious salmon anemia virus-*Aeromonas salmonicida-Vibrio anguillarum-ordalii*-bacterin, killed virus

Yersinia ruckeri bacterin

Flavobacterium columnare bacterin

Flavobacterium columnare vaccine, avirulent live culture

Infectious pancreatic necrosis virus, killed virus

Infectious hematopoetic necrosis virus (IHNV), DNA vaccine

Generic Names:

Bacterin, Avirulent Live Culture, Inactivated/Killed Virus, Live Attenuated Virus, DNA vaccine

Trade Names:

Apex-IHN, Aquavac-COL, Aquavac-ESC, Furogen Dip, Lipogen Forte, Renogen, Forte V II, Ermogen, Fryvacc1, Vibrogen 2

CAS Numbers

Chemical Abstracts Service does not cover veterinary biologicals.

Other Codes:

USDA Animal Plant Health Inspection Service (APHIS): 1443.20, 1531.00, 17F1.00, 265H.01, 4A45.20, 2035.02, 2138.02, 2974.00, 2858.03, 2638.00

Summary of Petitioned Use

A petition was submitted on June 12, 2012, by the Aquaculture Working Group requesting allowance of vaccines, including vaccines produced by excluded methods for example, DNA vaccines for use in aquatic animals provided that these vaccines meet requirements defined by 9 CFR Chapter 1, Subchapter E, parts 101-124, Viruses, serums toxins, and analogous products; organisms and vectors. The petition requested that vaccines (biologics) for aquaculture be added to the National List as follows

§ 205.611 Synthetic substances allowed for use in organic aquatic animal production

(x) As disinfectants, sanitizers, and medical treatments as applicable.

(y) Biologics – Vaccines.

As required by the Organic Foods Production Act, the National Organic Standards Board has the responsibility to review each application for inclusion of a synthetic substance(s) in the National List. The NOSB has requested a full technical evaluation report for vaccines for aquaculture to support their decision-making.

Characterization of Petitioned Substance

Composition of the Substance:

Vaccination against infectious disease has been practiced for many decades and proves to be one of the most cost efficient means of reducing animal suffering and economic loss due to bacterial and viral infections (Horzinek et al., 1997). A vaccine contains or produces a substance(s) called an antigen(s) that stimulates an innate and/or adaptive immune response in an aquatic organism against a particular pathogen. The immune response enables protection from disease and resistance to future infection. Primitive aquatic species only have innate immune systems that respond non-specifically to pathogens and have limited memory of prior antigen exposure. More advanced vertebrate species possess both innate and adaptive immune systems with an extensive memory of prior infections.

Fishes are the most primitive vertebrates possessing a developed immune system including an adaptive immune response. Physiologically, the fish adaptive immune response results in an expansion of a population of antibody producing cells called B-lymphocytes. Antibodies produced by B-lymphocytes are specialized proteins that self-configure to specifically bind to a site(s) on the cognate antigen, and subsequently process it to be neutralized and removed. The immune response, a complex process, also stimulates immune functions and hematopoietic lymphoid and myeloid tissues playing roles in pathogen removal and neutralization, homeostasis, maintaining a memory of the infection and restoring functions lost during infection. The main lymphoid organs of fish are thymus, anterior (head)-kidney, spleen and blood tissue. The anterior-kidney is unique to fish. It contains a large population of lymphocytes and actively produces antibodies. The anterior-kidney is a major site of erythroid, lymphoid and myeloid cell production and antigen trapping (Deivasigamani, 2007).

Most of the vaccines approved for use by the United States Department of Agriculture (USDA) for fish are produced by conventional methods starting with natural pathogens grown in culture (van Oirschot, 1997). For inactivated vaccines, inactivating agents, i.e. formaldehyde, β-propriolactone and ethyleneimine are used to reticulate pathogen proteins that interact with cellular receptors and block nucleic acid replication. Disadvantages for killed vaccines are the potential for immunosuppressive passenger antigens, toxic reactions caused by immune-enhancing adjuvants, reduced immunogenicity due to denaturation of proteins and systemic reactions.

Modified live vaccines are prepared from one or more viruses, bacteria or parasites of attenuated virulence or natural low virulence for the target species. Pathogens are attenuated with heat, serial passage in cell culture, culture under abnormal conditions or genetic manipulation (Desmettre and Martinod, 1997). Of conventionally prepared vaccines, modified live vaccines stimulate the best immune response. However, hazards including residual virulence, virulence in immune-compromised vaccinates and reversion to virulence due to natural genetic recombination must be monitored and sometimes make modified live vaccines difficult to license.

A number of the known pathogens and parasites in aquaculture and availability of vaccines for them are described in Table 1. Pathogens causing these diseases and parasitic infections are frequently immunoevasive or immunosuppressive: they are able to avoid or reduce the fish's immune response and proliferate. Thus in many cases, inoculation with conventional vaccines consisting of whole pathogens or parasites, live or killed may not result in immunization. In order to develop vaccines for all of the fish diseases, veterinary immunologists have begun to use biomolecular approaches to 1) identify specific molecules from pathogens capable of stimulating sterilizing immunity, 2) develop effective methods for producing these antigens and 3) establish strategies for delivering antigenic vaccines in the absence of immunoevasive or immunosuppressive substances. Most often the immunizing principle is found in a polypeptide that is strongly recognized by antibodies. Peptides or DNA encoding specific peptides have been used in the development of recombinant vaccines: protein subunit vaccines, live recombinant vaccines and DNA vaccines.

Table 1 Known Fish Diseases[1]				
Disease	*Causative agent*	*Major affected fish species*	*Country/region*	*Commercially available vaccine(s)*
Bacterial Diseases				
Enteric redmouth (ERM)	*Yersinia ruckeri*	Salmonids, primarily rainbow trout	North America, Europe, South, America	yes
Vibriosis				
- Vibriosis	*Vibrio anguillarum, V. ordalii*	Widespread in marine fish: salmonids, cod, halibut, sea bass, bream, amberjack, yellowtail	Worldwide, Japan, North, America	yes
- Hitra disease	*V. salmonicida*	Atlantic salmon	Norway, Faroe Islands	yes
Furunculosis	*Aeromonas salmonicida subsp.salmonicida*	Salmonids	Canada/USA, Europe	yes
Atypical furunculosis	*Aeromonas salmonicida*	Salmonids Various FW/SW species	Globally	yes no
Bacterial kidney disease	*Renibacterium salmoninarum*	Salmonids	North America, Europe, Japan, Chile	yes yes
Enteric septicemia	*Edwardsiella ictaluri*	Catfish	Southeastern United States, Canada Asia	yes
			Asia	no
	E. tarda	Catfish, Eel, hirame	Japan	no no
Motile aeromonid septicemia	*Aeromonas hydrophila, A. caviae, A. sobria*	Catfish, cyprinids, salmonids	Asia, Europe, United States	no
Pasteurellosis	*Pasteurella piscicida*	Ayu, yellow tail, sea bream, sea bass, carp	United States, Japan, Europe, Taiwan, Province of China	yes no
Bacterial cod-water disease	*Lyto psychrophilus*	Salmonids	United States, Europe, Japan	
Streptococcus infections	*Streptococcus* spp.	Yellow tail, rainbow trout, ayu, tilapia, bass, bream	United States Chile Taiwan Province of China, Japan,	no no yes
Tuberculosis	*Mycobacterium marinum, M. fortuitum, M. chelonae*	Snakehead, tropical aquarium fish, sea bass, wide variety of other species	Southeast Asia, Japan, Europe	no
Nocardiosis	*Nocardia asteroides, N. Kampach*	Tropical aquarium fish, yellow tail, rainbow trout and brook trout	Spain, Japan, Canada	no
Salmonid rickettsial septicemia	*Piscirickettsia salmonis*	Salmonids	Chile, Taiwan Province of China, Ireland	yes
Epitheliocystitis	*Chlamydia-like* organisms	Wide variety of species	North America, Southeast Asia, Europe, South Africa	no
Clostridial infections	*C!ostridium botulinum*	Salmonids	Europe, United States	no
Columnaris disease	*Flexibacter columnaris, F. maritimus*	All freshwater species, bream, bass, turbot, salmon	North America, Asia, Europe, Japan	yes
Enterococcus infection	*Entrococcus serrolcida*	Yellow tail	Japan	no
Bacterial gill disease	*Cytophaga* spp., *Flexibacter* spp. *Flavobacterium bronchiophilia*	Wide variety of species	North America, Japan, Europe	no
Flavobacteriosis	*Flavobacterium psychrophilum*	Salmonids, FW	Chile, Canada/USA (West)	yes
Rainbow trout fry syndrome	*Flavobacterium psychrophilum*	Salmonids, FW	Europe, Canada/USA (West)	no
Ulcerative septicemia	*Pseudomonas* sp.	Eels and others	Japan	

Table 1 (cont.) Known Fish Diseases[1]				
Disease	*Causative agent*	*Major affected fish species*	*Country/region*	*Commercially available vaccine(s)*
Wound disease	*Moritella viscosa*	Salmonids	Northern Europe	yes
Lactococciosis	*Lactococcus garviae*	Rainbow trout Amberjack, yellowtail	Italy, France UK, Japan	yes yes
Viral Diseases				
Infectious pancreatic necrosis, other aquatic birnaviruses	Birnavirus (ds RNA)	Salmonids Sea bass, sea bream, turbot, Pacific cod	Globally	yes yes
Pancreatic disease virus	Alphavirus	Salmonids	UK, Ireland, Norway	yes
Viral haemorrhagic septicemia	Rhabdovirus	Salmonids	Japan, North America, Europe	no
Infectious hematopoietic necrosis virus	Rhabdovirus	Salmonids	Japan, North America, Europe	yes[2]
Infectious haemorrhagic necrosis	Rhabdovirus	Snakehead, carp, barbs	Japan, Taiwan Province of China, Canada, North America	yes
Infectious salmon anemia	Orthomyxovirus	Atlantic salmon	Norway, Canada/USA, UK	yes
Viral nervous necrosis/SJNNV and several other betanodavirus	Betanovirus	Several marine fish species, e.g., sea bass, groupers, barramundi, halibut	Globally	no
Iridoviral disease/RSIV	Iridovirus	Red sea bream, amberjack/yellowtail	Asia	yes
Channel catfish virus disease/CCV	Herpesvirus	Channel catfish	USA	no
Spring viremia of carp: /SVCV	Rhabdovirus	Mostly carp species	Europe, Canada/USA	yes
Grass carp hemorrhage disease/GCHDV	Aquareovirus	Grass carp	China	yes[3]
Parasitic diseases				
Sea lice	*Lepeophtheirus solmonis*	Marine-cultured salmonids	Northern circumpolar (Norway, Japan, Scotland, Ireland, Canada)	no
Proliferative kidney disease	Unidentified myxosporean extrasporgonic stage, PKX	Freshwater salmonids	United Kingdom, Europe, United States	no
Costiasis	*Jchthyobodo necotor*	Freshwater, non-host-specific fingerling fish especially affected. Also a saltwater form	Worldwide, 2-30°C	no
White spot	*Jchthyophthirius mulifiliis*	Freshwater, especially young fish; e.g. cyprinids, tilapiids, salmonids, ictalurids	Worldwide, 2-30°C	no
Trichodinids	*Trichodino* sp., *Tripartiello* sp. and others	Freshwater and marine non-specific salmonids flatfish (e.g. turbot) in culture	Worldwide, 4-25°C	no
Myxosporeans	A range of pathogenic species, e.g. *Myxobolus* spp., *Sphaerosporo* spp., *Kudoa* spp.	Freshwater and marine. All cultured fish	Worldwide	no
Microsporean	*Pleistophora* sp., *Glugeo* sp. and others	Freshwater and marine. One reported problem in flatfish	Worldwide	no
Fungal Diseases				
Icthyophoniaisis	*Ichthyophonus* spp.	Freshwater and marine species	Worldwide	no
Branchiomycosis	*Branchiomyces sanguinis, B. demigran*	Cyprinids, eels, freshwater tench, stickleback	India, Japan, Eastern Europe	no

Table 1(cont) Known Fish Diseases[1]				
Disease	*Causative agent*	*Major affected fish species*	*Country/region*	*Commercially available vaccine(s)*
Saprolegniasis	*Soprolegnia parasitico* - diclina complex	Cold, freshwater salmonids, catfish	Northern Europe, United States	no
Aspergillomycosis	*Asperilllus* spp	Tilapia	Worldwide	no
Epizootic ulcerative syndrome	Unknown (putative fungus)	Freshwater and brackish species	Australia, Southeast Asia	no
FW: Fresh water; SW: Salt water. [1]Adams et al., 1997; Sommerset et al., 2005 [2]DNA vaccine available, previously available inactivated virus vaccine is no longer commercially available [3] Previously available but may not be in use today				

Although recent vaccine products produced with excluded methods may be named so their method of production and origin is recognizable, it may not always be possible to differentiate them solely upon the true name assigned by the Center for Veterinary Biologics of the USDA, Animal and Plant Health Inspection Service (CVB). CVB has recently begun updated its naming convention for vaccines containing recombinant organisms (Table 2—APHIS, 2013b). Historically, naming of recombinant vaccines has been variable and names previously assigned to vaccines containing recombinant organism may not be accurate.

Table 2 Current APHIS CVB guidance for true names for vaccines containing recombinant organisms[1]

Antigen/Gene Expression System	Naming Convention
-antigen expressed in a recombinant system	*-subunit vaccine*
-gene deleted or inserted into an organism producing a recombinant seed	*-the recombinant is named the same as the conventional product.*
-a foreign gene is inserted into an expression vector and a) the final product contains both the vector and the expressed foreign protein, and b) there is a label claim for the vector as well as the insert protein	*-true name includes the identity of the vector; the inserted genes are reflected in the true name, and the identity of the vector appears as a modifier*
- a foreign gene is inserted into an expression vector and a) the final product contains both the vector and the expressed foreign protein but efficacy against disease caused by the vector has not been established	*-the identity of the vector is not currently included in main part of the true name.*
-the organism has had essential genes deleted and the foreign inserts are necessary for replication competence	*-chimera*
-the organism that receives the foreign inserts is replication competent without the inserts	*-vector*

[1]The true name is based on the entities for which there is a biological claim.

Source or Origin of the Substance:

The first studies on the immune response of fish to *Aeromonas salmonicida*, the causative agent of furunculosis were published in the mid 1930s and passed unnoticed due to the rise at that time in interest and subsequent use of antimicrobial compounds, such as antibiotics, nitrofurans, sulfa compounds, calomel and others. Immediately following World War II, husbandry served as the primary means of disease control in aquaculture. When this failed, the main recourse was chemical therapy (Evelyn, 1997).

Aquaculture subsequently emerged as a revolution in agriculture of global importance (Duarte et al., 2007). About 430 (97%) of the aquatic species presently in culture have been domesticated since the start of the 20th century, and an estimated 106 aquatic species have been domesticated over the past decade. This growing aquaculture industry brought both the specter of profit limiting disease and a renewed interest in vaccination (Sherwood, 1993). Over time the disadvantages of using chemical therapy became apparent. These were high cost, short term protection, high cost of getting new drugs approved by the USDA, requirements for adequate drug clearance before treated fish could be marketed and the development of drug resistance (Evelyn, 1997). The development of the first commercial fish vaccines for enteric red mouth disease (ERM—Ross et al., 1966) and furunculosis (Harrell et al., 1976) occurred in late 1960s and 1970s. Since then, billions of fish have been vaccinated against a number of economically important pathogens.

Properties of the Substance:

Vaccination is useful for a number of reasons, e.g. preventing or significantly reducing clinical signs and chronic conditions in the host during and after pathogenic infection; preventing viral or bacterial shedding by the host; preventing the spread of virus by secondary direct infection of another host or indirect transmission through a carrier and preventing epizootics. Vaccines properties vary depending on the pathogen for which it has been made and the type of vaccine.

Vaccines are best administered as early as possible in the life cycle of fish in order to protect them through the most vulnerable stages of development. Four methods are used for vaccinating fish: injection, immersion, spray and oral. Each has its advantages and disadvantages (Table 3). Injecting vaccine on a commercial scale is very labor intensive, requiring crews of people working continuously to immunize fish. The direct or dip immersion method is the most widely used means of immunizing fish. It is very economical on smaller fish (<l0-15 g), though stressful. Fish are exposed for a minimum of 20 seconds to well-aerated diluted suspensions of the vaccine and returned to holding tanks, etc., where they are held long enough to develop adequate levels of protective immunity. Spray vaccination adequately immunizes fish as long as they are exposed for at least two seconds. Unfortunately fish must be handled, potentially causing physical damage and stress. Many modifications of the spray method exist. Another route of immunization is the oral route. Fish are fed inactivated bacterial suspensions in the form of a paste or liquid suspension either coated onto or milled into feed (Newman, 1993).

Table 3. Comparison of various routes of vaccine administration*

Route of Immunization	Fish Weight	Advantages	Disadvantages
Injection Typically 0.1-0.5 ml intraperitoneally (IP), intramuscularly (IM), or subcutaneously (SC)	>15 g	Highest levels of protection; very economical for larger fish; allows for ready administration of adjuvants and chemotherapeutants; semiautomated injection technology exists	Very labor intensive; fish must be individually handled resulting in stress; hazardous to persons doing injecting
Immersion Dip-l:lO-1:lOO dilution for 20-120 s Bath-l:lOO-1:lOOO for 120 s-30 min	1-5 g	High levels of protection; not as stressful as injection; most widely used method; semiautomated technology exists; allows in situ immunization in hatchery troughs, holding tanks, transport vessels, and net pens	Fish must be handled; weight per unit volume limitations make it uneconomical for larger fish; labor intensive; adjuvant delivery problematic
Spray (shower)	1-5 g	High levels of protection; 3-10 times the poundage per unit volume of immersion; semiautomated technology exists	Fish must be handled; labor intensive; specialized machinery required
Oral (by feed or per OS)	1-5 g	No handling of fish required; moderate, variable levels of protection; no handling of vaccine or machinery is required	Levels of protection variable and are not as great as that provided by other methods; probably best as a secondary or boost vaccination

*Newman, 1993, Kibenge et al., 2012

There are two broad groups of conventional vaccines: inactivated and modified live. Inactivated vaccines contain microorganisms and viruses rendered non-infectious by inactivation. Viruses do not fit the classical growth, reproduction, irritability, metabolism (GRIM) definition for an organism, but are important in disease and vaccinology. When the inactivated microorganism is bacterial, the resulting vaccine is called a bacterin. Inactivated vaccines produced from the supernatant of a bacterial culture or from an inactivated toxin are called toxoids. Formaldehyde is the most widely used agent for inactivating viral, bacterial and parasitic pathogens. It not only kills the microorganism, but denatures and preserves its proteins. Formaldehyde is removed during the inactivation process once the pathogen is inactivated. Effective use of inactivated vaccines requires the addition of adjuvants that non-specifically enhance the innate immune response to a given antigen boosting protective immunity (van Oirschot, 1997). Adjuvants are produced from a wide array of substances including oil water emulsions, aluminum containing compounds and various chaperoning proteins such as the 70 kilodalton heat shock protein. A major drawback for adjuvant use is the production of a site specific reaction or toxicity: the potential of some adjuvants to cause a lesion at the site of injection. Injection site reactions can render meat unsuitable for consumption reducing its value.Modified live vaccines are produced in a number of ways. Classically, modified live vaccines for viruses, bacteria and parasites are produced by growing the organisms *in vitro* or in an alternate host through many generations, and selecting for naturally occurring mutants that no longer cause disease, but are still replicative. Recently, live bacterial vaccines have been developed by selecting for a specific drug resistant over a number of passages and assessing for residual virulence (Pridgeon et al., 2013). Advantages include delivery by a number of routes, good presentation of antigens, since the organism is growing in the

host, and longer acting as a result of cellular potentiation of the immune response (Horzinek et al, 1997). Live vaccines may still be immunosuppressive. Furthermore, because the attenuation mutations in some conventional live vaccine are produced at random and not determined at the molecular level, it is impossible to predict where they will occur and under what circumstance the may revert to virulence. Vaccines rendered avirulent with a single point mutation can revert to virulence in one passage.

Biomolecular methods such as restriction enzyme mapping, the polymerase chain reaction (PCR), DNA sequencing and microarray analysis have facilitated antigen discovery, construction of novel candidate vaccines and assessments of vaccine efficacy, mode of action and host response (Kurath, 2008). These methods also provide strategies to identify alter and delete the virulence genes of various pathogens. For example in bacteria deleting genes involved in adhesion, toxin production or any one of the physiologically important biosynthetic pathways can alter the organism's ability to cause disease. Viral attenuation through deletion of virulence genes provides opportunities to insert heterologous genes for other pathogens to convert the once virulent virus to a functional live vaccine (Babiuk, 1997c). Biologics produced with biomolecular methods include subunit and monoclonal antibody vaccines, recombinant modified live vaccines, and DNA vaccines. An exemption under section 205.105 allows vaccines from excluded methods to be considered for addition to the National List.

Subunit vaccines consisting of proteins and glycoproteins capable of inducing a protective immune response are potentially more economical and safer than conventional killed or live vaccines. Subunit vaccines lack immune-interfering or immunoevasive substances that can be present in whole organism vaccines and are not likely to revert to virulence. They are also better suited for multicomponent vaccination, and provide a way to differentiate vaccinates from infected fish. Their production is facilitated by identifying genes encoding the protein or glycoprotein of interest and expressing these genes in an appropriate expression system or using synthetic peptide technology (Babiuk, 1997a). Semi-synthetic and synthetic glycoconjugate vaccines have been used in other organisms for protection against bacteria. These may one day offer a non-excluded vaccine for organic aquaculture (Constantino et al, 2011)

Early vaccine research showed the potential of using heterologous viruses to induce immunity against a pathogen with a non-virulent organism of distinct origin. Historic work by Edward Jenner and Louis Pasteur demonstrated the immunization potential of Vaccinia virus for smallpox (Babiuk, 1997b). Bacterin immunization against *Streptococcus iniae*, a bacterial pathogen of Nile Tilapia, *Oreochromis niloticus* is complicated by immunoevasion resulting from the emergence of new bacterial serotypes. Shoemaker et al. have shown that it is possible to immunize against *S. iniae* with a heterologous bacterin (2010). Bacterial kidney disease in salmonids is caused by the bacterium, *Renibacterium salmoninarum*. Renogen® containing the non-virulent bacterium, *Arthrobacter davidanieli* is used as a live vaccine for this disease, because the vaccine bacteria and the pathogen share a conserved protein that serves to stimulate immunity against the pathogen.

Antibodies, the first responder to antigens, can neutralize pathogens. Monoclonal antibodies can be produced commercially *in vitro* and used for this purpose. The method for producing monoclonal antibodies requires the production of a hybridoma cell line requiring cell fusion. Essentially, a B-lymphocyte from the spleen of an immunized animal identified as producing neutralizing antibody is fused *in vitro* to cells capable of constitutive antibody production. Cells from the fusion are screened for one that produces the pathogen neutralizing antibody. Once identified, and isolated the cell is expanded into a cell line. The cell line produces the antibody, which is purified and potentially may be used to directly treat infected animals. Although this technology is currently very expensive, its importance in diagnostics and continuous improvement of production technologies will potentially bring production costs down in the future (Lorenzen et al., 1990). Antibodies may also be used for the production of anti-idiotypic vaccines. These vaccines are produced by producing antibodies against neutralizing antibodies. The anti-idiotype mimics the original antigen. Anti-idiotypic vaccines are useful against tumors (Meloen, 1997).

Bacterial plasmids are natural circular chromosomes composed of DNA that replicate independently of the bacterial cell and provide a natural means for bacteria to conjugally transfer genetic information between bacteria (Meyer et al., 1975). They may number in the hundreds per bacterial cell and naturally maintain specific drug resistance genes. The potential of bacterial plasmids was developed with the introduction of cloning specific DNA sequences (Bolivar et al., 1977). Bacterial cloning enabled the scientific study of DNA sequences from many organisms (Sanger and Coulson, 1975). Enzymes isolated from bacterial cells were

used for both cutting DNA at specific sequences and ligating it back together with new sequences inserted. Amplification in bacterial culture of this newly introduced DNA sequence on the plasmid provided sufficient DNA for sequencing chemistry. With sequencing, a better understanding of how bacteria express proteins developed and specific DNA sequences required for expression were identified and subsequently introduced into plasmids to provide the machinery for bacteria to express exogenous proteins. This work extended to higher organisms that maintain plasmids such as yeasts, and has reached a third generation with the successful construction of a DNA vaccine (Tang et al., 1992). DNA vaccines are made from purified bacterial plasmid DNA. DNA sequences encoding pathogen proteins are inserted into bacterial plasmids for antigen expression. Plasmid DNA containing antigenic inserts can be purified from bacterial cultures inexpensively.

When injected into muscle cells, both DNA from the DNA vaccine and its expressed antigen are recognized by the innate immune system. The antigen protein expressed by the DNA vaccine is also recognized and processed as part of the adaptive and cellular immune response. B-cells proliferate when they recognize their cognate antigen presented on the surface of an antigen presenting cell and produce neutralizing antibodies against the pathogen. Other immune cells are involved in stimulating a long lasting cellular immune response in the host (Tonheim et al., 2008). DNA vaccines against infectious diseases have several benefits including low cost, ease of production and improved quality control, heat stability, identical production processes for different vaccines, and the possibility of producing multivalent vaccines (Gilund et al., 2008). Only two DNA vaccines are internationally licensed for veterinary use, one is for vaccination against West Nile Virus in horses and the other is against Infectious Hematopoietic Necrosis (IHN) virus in Salmon. A Federal Register Notice dated December 31, 2013, announced a 30 day comment period for the anticipated authorization by the USDA for shipment and sale of the DNA vaccine for IHN in the United States (APHIS, 2013c). This will be the first DNA vaccine licensed for aquaculture in the US.

Specific Uses of the Substance:

Local pathogens, pathogens transferred as a result of international trade, inadequate farm management, environmental factors, and poor water quality are the main causes of disease in aquaculture. In spite of efforts to eradicate fish disease, unforeseen outbreaks can cause serious losses in production, impacting the livelihoods and food security of aquatic farmers. Disease control by vaccination offers one option to consider when there is reason to doubt the practicability or likely success of eradication measures (Hill, 2005). Vaccines for many fish diseases have been developed, but there are a number of diseases for which there is no vaccine.

The economics of vaccination are important, since no vaccine is 100% effective. Some fish will not be protected and some disease may still occur. However, an effective vaccine program will not only reduce economic loss through reduced mortalities, but should also alleviate reduced growth rates due to infection (Ellis, 1997a). Several examples of important fish bacterial disease vaccines follow and Table 1 provides the availability of vaccines for the major bacterial fish diseases.

Vaccines should be administered only to healthy fish. They are one component of a complete fish health program (U.S. Fish and Wildlife Service, 2011). Unlike antibiotics, vaccines rely on a healthy fish immune system to be effective and are used to prevent the occurrence of a specific disease outbreak (Yanong, 2008).

Vibriosis is one of the most serious bacterial diseases of farm raised fish affecting Pacific salmon, Atlantic salmon, rainbow trout, turbot, sea bass, sea bream, striped bass, cod, Japanese and European eel and ayu. There are at least eight species of *Vibrio* which have been associated with diseases of fish. Vaccines are available for four of them: *V. anguillarum*, *V. ordalii*, *V. salmonicida*, and *V. parahaemolyticus*. All vaccines produced commercially for Vibriosis are bacterins (inactivated bacterial culture). Some may be administered by immersion, but the most effective immune response is achieved by injection at about 1 month of age. Various adjuvants are added to the vaccines to improve immunogenicity. USDA approved vaccines include Furogen, Lipogen Forte, Forte IV, and Vibrogen 2 (US Fish and Wildlife Service, 2011).

Enteric redmouth (ERM) disease is caused by the pathogen *Yersinnia ruckeri*. It is primarily a disease of freshwater fish including rainbow trout and Coho salmon. The ERM vaccine, an inactivated whole bacterial cell vaccine was one of the first produced for aquaculture. It is effective both by immersion or injection. In

the case of ERM and rainbow trout, if immersion vaccination of trout resulted in 1% more fish reaching the market, the cost of the vaccine can be recovered (Ellis, 1997).

Furunculosis is an important disease of wild and farmed salmonids throughout the world, except South America (Ellis, 1997b). The bacterium that causes furunculosis, *Aeromonas salmonicida*, is immunoevasive. Phagocytic cells called macrophages which are normally a first line of defense in fish disease response cannot kill *A. salmonicida* in a naïve host. However, vaccination with a whole cell bacterin that has been emulsified in an oil based adjuvant overcomes bacterial immunoevasion and is effective in protecting the fish. An important issue with oil based adjuvants is the production of unsightly granulomatous lesions at the injection site. In fact, many producers avoid the use of this type of vaccine which must be injected because of the side effects.

Enteric septicemia of catfish (ESC) is a major disease problem facing commercial catfish production. The etiological agent of ESC is *Edwardsiella ictaluri*. Another agent *Flavobacterium columnare* is also economically important. Vaccines containing bacterins for these pathogens have not been very effective, because they are immunoevasive. Live attenuated vaccines have emerged as the best choice for vaccination. Catfish fry 7-10 days old can be effectively immunized by immersion for either of these agents (Sommerset et al., 2005).

Vaccination is the most effective method of controlling viral disease and commercial vaccines are available for fish. Most of the virus vaccines available for aquaculture are inactivated/killed viral vaccines or recombinant subunit proteins (Salgado-Miranda et al., 2013). Table 1 provides a list of the major viral fish diseases and their respective vaccine availability.

Inactivated vaccines have been used for infectious pancreatic necrosis in Atlantic salmon and for grass carp hemorrhagic disease. These rely heavily on the extent of treatment with chemicals such as formaldehyde, ethyleneimine or β-propriolactone to preserve immunogenicity but prevent virulence. Inactivated virus vaccines can be administered orally, by immersion or by injection depending on the particular vaccine. Some must be administered with an adjuvant for a good response. In this case the vaccine is injected. Inactivated vaccines are the most expensive to produce.

Modified live vaccines are desirable, and highly effective for closed systems. However, the virus is still capable of infection. These vaccines have not usually been considered acceptable due to the environmental risk that non-virulent viruses could revert to virulent forms or that attenuated viruses that are not virulent in vaccinated species could prove virulent to other species in open systems (Salgado-Miranda et al., 2013).

The aim of vaccination and developing improved vaccines is not only to reduce economic losses, but also to prevent mass destruction of large numbers of infected or potentially contagious animals; to prevent the transmission of infectious disease to humans; to contribute to the health and welfare of domestic and wild animals and to protect the environment (Pastoret et al., 1997). An ideal vaccine is economical; easy to produce and administer; capable of inducing a strong, lasting and protective immunity in a single dose; safe for fish, with minimum side effects; noninvasive and able to be administered early; stable at ambient temperature; and without negative environmental impact (Salgado-Miranda, 2013).

Approved Legal Uses of the Substance:

The United States Department of Agriculture (USDA), Animal Plant Health Inspection Service (APHIS), Center for Veterinary Biologics (CVB) regulates veterinary biological products produced in, imported into, transported through or exported from the United States. This ensures that products are pure, safe, potent and efficacious, and not worthless, contaminated, dangerous or harmful. The authorities and procedures for US regulation are described in the Virus Serum Toxin Act of 1913 (amended in 1985) and title 9 parts 101-121 of the US Code of Federal Regulations (Birnbaum, 1997). There are other documents produced by the USDA APHIS CVB supporting biologics regulation, guidance, use and approval. The licensing process for vaccines in the US is rigorous requiring both validation of the vaccine and its adjuvants and inspection of the manufacturing establishment. The USDA has approved the use of one DNA vaccine for fish and aquaculture. A Federal Register Notice dated December 31, 2013 has been sent out for final comment (APHIS, 2013c).

Action of the Substance:

Living organism are reactive systems, not preprogrammed. They respond in parallel to many concurrent inputs for example DNA code, structural proteins, enzymes, carbohydrates, lipids, intracellular signals, hormones and other molecules that play key roles in both forming and informing the system (Cohen and Harel, 2006). In responding to infection, fish organize the immune system and inflammation in a way that attempts to maintain, heal, and regenerate damaged tissue (Raz et al., 2001). Pathogenesis and immune response vary between fish and their pathogens. This is often an issue in both bacterial and viral infections because there is often significant variation in antigenicity within species. Generally, vaccination minimizes pathogenesis by stimulating the immune system in advance of disease, so that vaccinates have already undergone the first steps in the immune response prior to encountering the pathogen.

Combinations of the Substance:

Many chemicals are used in the preparation of fish vaccines. Both bacterial cell culture and animal cell culture used to propagate bacteria and viruses require well-defined media that is usually not sourced organically.

Most fish vaccines are chemically inactivated. Formaldehyde, ethyleneimine and β-propriolactone are commonly used for inactivation. None of them is included in the National List; however, other veterinary vaccines that have been inactivated using these chemicals are included.

Adjuvants and/or immunostimulants are added to vaccines to improve antigenicity. The use of oil adjuvants in injectable vaccines has been approved by the USDA for *A. salmonicda*, *Y. ruckeri* and *Vibrio* species. Adjuvants are necessary for generating an effective immune response, because they prime the innate immune system, leading to an expanded adaptive immune response. Adjuvants and immunostimulants are not considered excipients.

Polyvalent vaccines are combinations of several antigens, mixed together in optimized ratios to produce strong immunity to all of the components. Antigens can be bacterial or viral, killed or attenuated. Some vaccine combinations are effective; however, antigen competition, and antigen interference, may prevent other combinations. Vaccines combinations should always be used under veterinary supervision, because incompatibility between vaccines from different sources may result in adverse events, i.e., injection site reaction or return to virulence.

Status

Historic Use:

The National Organic Program (NOP) and the National Organic Standards Board (NOSB) have acknowledged the importance of vaccines in preventing disease. The USDA organic regulations require that the producer establishes and maintains preventive livestock health care practices including administration of vaccines and other veterinary biologics (7 CFR 205.238(a)(6)). NOSB acknowledged that the rise in vaccines developed using excluded methods made sourcing conventional vaccines increasingly difficult; however, in the special case of vaccines, jeopardizing the health and safety of potentially millions of animals or the public by prohibiting selected vaccines developed with excluded methods was not considered an option (NOSB, 2009). Ensuring that livestock producers are not hindered in preventing disease in their herds, NOP changed the wording of section 205.105(e) and the regulation in regards to vaccines. Pertinent to this discussion is the history of fish vaccines which were first described in 1939, but ignored until 1970, due to the rise in convenient chemotherapeutic methods for disease control (Evelyn, 1997).

Current biomolecular methods are also effective for the development of cost effective vaccines, because the same technologies used to determine the molecular basis for disease have been applied to vaccine development. Canada has already licensed a DNA vaccine for infectious hematopoietic necrosis virus in salmonids. This vaccine will also be available for fish in the United States (APHIS, 2013c). Apex-IHN is the first and only DNA vaccine licensed for commercial use in aquaculture. It was licensed in Canada in 2005

by Vical, a division of Novartis Animal Health, Inc. because an efficacious conventional vaccine for IHN epizootics British Columbia from 2001-2003 was not available (CFIA, 2005).

Organic Foods Production Act, USDA Final Rule:

The OFPA describes organic wild seafood in §6506(c) recommending that the USDA consult with the US Department of Commerce to accommodate US Fisheries (7 U.S.C. 74, 2013). However, NOSB's recommendations are limited to aquaculture. The OFPA does provides an exemption for vaccination of livestock in § 6509(d)(1)(C). The National Organic Program Final Rule defines vaccines as biologics (USDA, 2013) consistent with 9 CFR 101-121, Subchapter E—Viruses, Serums, Toxins, and Analogous Products; Organisms and Vectors. In addition, an exemption for vaccination is provided in 7 CFR 205.105(e), that refers back to 7 USC 6517-6518, whereas the vaccine is not harmful to human health or the environment; is necessary for livestock production because there is no alternative; and fits with organic farming. In addition, the reference (§6518) provides for further evaluation by the National Organic Standards Board (NOSB). Section 205.238 of the USDA organic regulations on livestock health care, requires livestock producers establish preventive health care practices including the administration of vaccines, even in the absence of illness (§ 205.238(a)(6) and (c)(2)).

International

Canada - Canadian General Standards Board Permitted Substances List –

The Canadian General Standard for Organic Production Systems (CGSOP) defines vaccines as veterinary biologics and mandates the establishment and maintenance of preventative livestock health care practices, including the administration of vaccines in accordance with the standard when it has been documented that the targeted diseases are communicable to livestock on the enterprise and cannot be combatted by other means. The CGSOP permits vaccines to be used that have been grown on genetically engineered substrates but are not themselves a product of genetic engineering, as specified in CAN/CGSB-32.311, Organic Production Systems — Permitted Substances Lists (PWGSC, 2011a). The Canadian General Standards Board (CGSB) Permitted Substances List for Livestock Production classifies vaccines as health care products and restricts vaccines to those that have been grown on genetically engineered substrates but are not themselves a product of genetic engineering provided that there is documented evidence that the targeted diseases are communicable to livestock on the enterprise and cannot be combated by other means, and an analogous vaccine grown on a substrate not produced from genetic engineering is not commercially available and a reasonable search of veterinary suppliers has been conducted (PWGSC, 2011b).

The CGSB is in the process of developing a new national standard for organic aquaculture. It is sponsored by the Canadian Department of Fisheries and Oceans. A standard has recently been published by the Standards Council of Canada on aquaculture, CAN/CGSB-32.312-2012. It does not currently fall under the scope of Canada's Organic Products Regulations or Canada's trade equivalencies for organic products with the United States or European Union. The standard will be reviewed and amended within five years, and regulation and enforcement provisions will be sought.

CODEX Alimentarius Commission, Guidelines for the Production, Processing, Labelling and Marketing of Organically Produced Foods (GL 32-1999) - ftp://ftp.fao.org/docrep/fao/005/Y2772e/Y2772e.pdf

The Codex Alimentarius Commission (CAC) defines organic livestock as any domestic or domesticated animal including bovine (including buffalo and bison), ovine, porcine, caprine, equine, poultry and bees raised for food or in the production of food. The CAC does not consider products of hunting or fishing of wild animals as part of this definition (CAC, 1999). Provisions for aquaculture are currently under review and have been sent to member nations of the Codex Alimentarius Commission and interested international organizations for comment on all aspects including possible implications of the proposed draft standard for their economic interests. The next meeting of the Codex Committee of Food Labelling in 2014 will consider these comments and whether to amend GL 32-1999 to include organic aquaculture (CCFL, 2013).

GL 32-1999 includes the following reference to vaccination of livestock: the use of veterinary medicinal products in organic farming shall comply with the following principles: a) where specific disease or health problems occur, or may occur, and no alternative permitted treatment or management practice exists, or, in cases required by law, vaccination of livestock, the use of parasiticides, or therapeutic use of veterinary

drugs are permitted. CAC also provides that all materials and/or the products produced from genetically engineered/modified organisms (GEO/GMO) are not compatible with the principles of organic production (growing, manufacturing, or processing) and therefore are not accepted under these guidelines (CAC, 1999).

European Economic Community (EEC) Council Regulation, EC No. 834/2007 and 889/2008

Regulation (EC) No 834/2007 provides for organic aquaculture. It provides an exemption for the use of GMO veterinary medicinal products; allows for the use of chemically synthesized allopathic products for animal disease and permits the use of immunological medicines.

Regulation (EC) No 889/2008 states that it does not apply to products originating from aquaculture, but encourages that this work will follow. In this regulation, veterinary treatment means all courses of a curative or preventive treatment against one occurrence of a specific disease including vaccination. This regulation provides that the preventive use of chemically-synthesized allopathic medicinal products is not permitted in EU organic farming. However, in the event of a sickness or injury of an animal requiring an immediate treatment, the use of chemically synthesized allopathic medicinal products should be limited to a strict minimum and with the exception of vaccinations, treatments for parasites and compulsory eradication schemes where an animal or group of animals receive more than three courses of treatments with chemically-synthesized allopathic veterinary medicinal products or antibiotics within 12 months, or more than one course of treatment if their productive lifecycle is less than one year, the livestock concerned, or produce derived from them, may not be sold as organic products, and the livestock must undergo a conversion period as mandated. Thus, vaccination is exempt in the EU organic rule for agriculture (The Council of the European Union, 2008).

Regulation (EC) No 710/2009 amends regulation 889/2008 to include organic aquaculture. The use of allopathic treatments is limited to two courses of treatment per year, with the exception of vaccinations and compulsory eradication schemes. However, in the cases of a production cycle of less than a year a limit of one allopathic treatment applies. If the mentioned limits for allopathic treatments are exceeded the concerned aquaculture animals cannot be sold as organic products (The Council of the European Union, 2009). Regulation (EC) No 710/2009 refers to Council Directive 2006/88/EC which covers disease control in aquaculture. This document references the OIE Aquatic Animal Health Code and the Manual for Diagnostic Tests for Aquatic Animals (OIE, 2013). (The Council of the European Union, 2006). Overarching is the incumbent potential for epizootic spread of disease in an aquatic environment, notwithstanding the transfer of disease in closed systems as a result of import. This document empowers the competent authority to control disease in aquaculture and requires vaccination against OIE listed diseases, unless the participating member state has been declared free of this disease.

Japan Agricultural Standard (JAS) for Organic Production

The Japanese Agricultural Standard for Organic Livestock Products provides for ethical biological drugs and veterinary drugs as specified by Article 1. 1 of the Ministerial Ordinance for Handling Biological Drugs and Veterinary Drugs by the Ministry of Health, Labor and Welfare (No. 4 of 1961—MAFF, 2012). Under JAS standards, livestock disease is prevented by strengthening resistance to disease, infection, prevention, through appropriate husbandry practices depending on livestock. However, disease can be alleviated without undo suffering and with the use of vaccines as required by law or veterinary prescription. No GMO vaccines are licensed under Japan's regulations.

International Federation of Organic Agriculture Movements (IFOAM) – http://www.ifoam.org/standard/norms/cover.html

The IFOAM organic animal management systems follow the principle of positive health including prevention of disease with vaccines. Vaccines are allowed when a vaccination is legally required, an endemic disease is known or expected to be a problem in the region and where this disease cannot be controlled by other management techniques. IFOAM's norms make an exception for vaccines derived from genetically modified organisms (IFOAM, 2012).

Soil Association

The Soil Association permits vaccination for specific known disease risks, but does not permit the use of genetically engineered vaccines in their organic standard (Soil Association, 2013).

Naturland Association for Organic Agriculture, www.naturland.de

Naturland was the first organization internationally to develop a standard for organic aquaculture. The Naturland standard does not permit the use of genetically modified products or their derivatives in organic aquaculture. However, it defers to veterinary supervision concerning issues of animal health and disease prevention (Naturland, 2013).

KRAV (Sweden)

KRAV encourages that prophylactic work be carried out, including effective vaccination against relevant infectious diseases, so that outbreaks of disease and use of drugs are avoided to the greatest possible extent. However; this organization prohibits the use of GMO vaccines (KRAV, 2013).

China

In China, aquaculture producers may use vaccine inoculation to prevent disease when there is the risk of certain diseases that cannot be controlled by other management technology, or where it is provided for in the state laws. Genetically engineered vaccines are prohibited except for national compulsory immunization vaccines (China, 2011).

Evaluation Questions for Substances to be used in Organic Crop or Livestock Production

Evaluation Question #1: Indicate which category in OFPA that the substance falls under: (A) Does the substance contain an active ingredient in any of the following categories: copper and sulfur compounds, toxins derived from bacteria; pheromones, soaps, horticultural oils, fish emulsions, treated seed, vitamins and minerals; livestock parasiticides and medicines and production aids including netting, tree wraps and seals, insect traps, sticky barriers, row covers, and equipment cleansers? (B) Is the substance a synthetic inert ingredient that is not classified by the EPA as inerts of toxicological concern (i.e., EPA List 4 inerts) (7 U.S.C. § 6517(c)(1)(B)(ii))? Is the synthetic substance an inert ingredient which is not on EPA List 4, but is exempt from a requirement of a tolerance, per 40 CFR part 180?

The substance falls into the category of a medicine. Vaccines for aquaculture are veterinary medicinal treatments acting to stimulate pathogen specific immunity in the absence of pathogenic infection.

Evaluation Question #2: Describe the most prevalent processes used to manufacture or formulate the petitioned substance. Further, describe any chemical change that may occur during manufacture or formulation of the petitioned substance when this substance is extracted from naturally occurring plant, animal, or mineral sources (7 U.S.C. § 6502 (21)).

All commercial vaccines for use in the United States must be produced by establishments that have received establishment licenses from the USDA. The licensing process includes inspections of the establishment's facilities to ensure that it is capable of producing vaccines that are safe, efficacious and free of contaminants. All of the commercial vaccines used in the United States must each be approved and licensed to ensure that they are safe and efficacious. A list of vaccines and bacterins licensed by the USDA for use in fish raised or sold in the US is provided in Table 4.

Most of the vaccines used for aquaculture are conventional killed vaccines against bacterial pathogens: bacterins. Bacteria for these vaccines are cultured in large culture vessels called fermenters under controlled conditions. An inactivant, usually formaldehyde, ethyleneimine or β-propriolactone is introduced to the culture, as it enters the stationery phase of growth. The culture is washed to remove the inactivant, and the dead cells are dispensed into aliquots and lyophilized for better storage. An adjuvant which is necessary to improve the immunogenicity of the bacterin may be included with the bacterin prior to administration. Adjuvants for fish are usually oil based emulsions or aluminum hydroxide.

There are several killed virus vaccines used for aquaculture in the United States. Viruses must be grown in host cells. The host cells of choice are usually fish cell lines from common carp or salmon. Cells are grown

in bioreactors containing nutrient rich medium. Cells may be pre-infected with virus or virus may be inoculated into cells as they reach sufficient density in culture. When the virus titer reaches an acceptable level, virus in the culture is inactivated using formaldehyde, ethyleneimine or β-propriolactone. The culture is filtered to remove cell debris, and washed to remove the inactivant. The inactivated virus is dispensed and usually lyophilized. An adjuvant which is necessary to improve the immunogenicity of the killed virus may be included with the killed virus vaccine prior to administration. Adjuvants for fish are usually oil based emulsions or aluminum hydroxide.

Modified live bacterial and viral vaccines are also grown in culture. Vaccines are dispensed and frozen or lyophilized for storage.

DNA vaccines are plasmids produced within bacteria in culture. DNA plasmids are isolated from bacterial culture by lysis of the bacterial cell and chromatographic purification of covalently closed circular DNA from the lysed culture. Native DNA is not alive or infectious, thus it does not need to be chemically treated or inactivated. Purified DNA is dispensed and lyophilized for better storage. An adjuvant which is necessary to improve the immunogenicity may be included with the DNA vaccine prior to administration.

Currently no other vaccine type is used for aquaculture. Although, other types of vaccines may be in use for other animal species.

Table 4 Vaccines and bacterins currently licensed by the USDA for use in the United States[1]

APHIS Product Number**	Vaccine Name	APHIS Description	Disease	Vaccination Route
1K11.00	Renogen	Arthrobacter Vaccine[1]	Bacterial Kidney Disease	Injection
1443.20	(not commercially available)	Cyprinid Herpesvirus Type 3 Vaccine, Modified Live Virus[1]	Spring Viremia of Carp	Immersion
1531.00	Aqua-Vac ESC	Edwardsiella Ictaluri Vaccine, Avirulent Live Culture[2]	Chanel Catfish Septicemia	Immersion
17F1.00	Aqua-Vac Col	Flavobacterium Columnare Vaccine, Avirulent Live Culture[2]	Columnaris Disease	Immersion
265H.01	Furogen Dip	Aeromonas Salmonicida Bacterin[1]	Furunculosis	Injection
2138.02	Lipogen Forte	Aeromonas Salmonicida-Vibrio Anguillarum-Ordalii-Salmonicida Bacterin[1]	Vibriosis	Injection
2974.00	FryVacc1	Flavobacterium Columnare Bacterin[1]	Columnaris Disease	Immersion
2858.03	Vibrogen 2	Vibrio Anguillarum-Ordalii Bacterin[1]	Vibriosis	Immersion
2638.00	Ermogen	Yesinnia Ruckeri Bacterin[1]	Enteric Redmouth Disease	Immersion
4A45.20	Forte V *II*	Infectious Salmon Anemia Virus Vaccine-Aeromona Salmonicida-Vibrio Anguillarum-Ordalii Salmonicida Bacterin[1]	Infectious Salmon Anemia-Furunculosis-Vibriosis-Combo	Injection
Provisional in the US	APEX-IHN	Infectious hematopoietic necrosis virus, DNA vaccine	Infectious hematopoietic necrosis virus	Injection

[1]US Fish and Wildlife Service, 2011

[2] The alphameric system used for product codes provides for a six-digit number of number-letter combination to be assigned to each product, i.e., the first digit denotes product types; second and third, group by agents; fourth, the viability of vaccine, (live, killed, modified live, etc.); fifth, substrates; and sixth, miscellaneous variables (APHIS, 2013a).

[3]Novartis International AG, Postfach CH-4002, Basel, Switzerland
[4]Merck Animal Health, Merck & Co., 556 Morris Avenue, Summit, NJ, 07901-1330, USA, +1-908-473-3349

Evaluation Question #3: Discuss whether the petitioned substance is formulated or manufactured by a chemical process, or created by naturally occurring biological processes (7 U.S.C. § 6502 (21)).

Vaccines are created by naturally occurring biological processes including cell culture and fermentation. For most aquaculture vaccines, an infectious agent is used as the immunogen. The infectious agent can be a virus, a bacterium, a fungus or a protozoan. Each of these requires a different culture system, but they are grown naturally to produce as much antigen as possible per unit volume of culture medium. Viruses are grown in cell culture, since they depend on a living host for replication. Bacteria are grown in fermenters.

In fish cell culture, cells are removed from fish tissue and enzymatically or mechanically disaggregated before cultivation. Several continuous fish cell lines have also been established. Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. Culture conditions vary for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing the following: a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals); growth factors; hormones; gases (O_2, CO_2) and a regulated physico-chemical environment (pH, osmotic pressure, temperature). Most cells are anchorage-dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture). Cell lines are usually cryopreserved by treating with the appropriate protective agent (e.g., DMSO or glycerol) and storing at temperatures below –130°C (cryopreservation) until they are needed. In vaccine production, this is a major advantage because batches of virus can be produced consistently and reproducibly from the same a batch of clonal cells. If the vaccine is to be killed, it is inactivated by the introduction of a preservative to the culture. This is usually followed by washing and filtering. If the vaccine is a modified live vaccine the final product is usually lyophilized and resuspended when needed. Immersion vaccines are resuspended in tank water. Injectable killed vaccines are usually administered with an adjuvant consisting of an oil emulsion or aluminum hydroxide.

Bacterial vaccines are grown in vessels containing culture medium under controlled conditions. Medium consists of digested meat or vegetable products and salt. More fastidious bacteria may require additional nutrients. Modified live vaccines may simply consist of the culture medium itself. Fish are usually vaccinated by immersion with modified live vaccines. Inactivated vaccines are treated with preservatives and may be adjuvanted, but can be delivered by either immersion or injection.

DNA vaccines, plasmids contained within in living bacteria, are also grown in fermenters. When the culture reaches stationary phase, the bacteria are lysed enzymatically and DNA is extracted. Plasmid DNA is isolated from cell debris and other DNA based on its ability to remain supercoiled. Plasmid DNA is stable as a lyophilized or frozen product. In the case of DNA vaccines, restriction enzymes must be used to construct the DNA plasmid so that it functions properly when used. These enzymes are derived from bacteria and fungi, or may be synthetically produced. DNA vaccines are made with or constructed using biomolecular methods. The exemption under section 205.105 allows vaccines from excluded methods to be considered for addition to the National List.

Evaluation Question #4: Describe the persistence or concentration of the petitioned substance and/or its by-products in the environment (7 U.S.C. § 6518 (m) (2)).

Killed vaccines do not persist in the environment or in the vaccinated fish. Once administered, the fish's antibodies and phagocytic antigen presenting cells begin the process of removing the vaccine and building an immune response. The vaccine is digested and excreted during this process.

Modified live vaccines are designed primarily for administration in closed systems or in isolation tanks, because their release into the environment may inadvertently cause a toxic reaction to wildlife and studies to determine the extent of this side-effect are very costly. There are currently three modified live vaccines licensed for commercial use in the US. These vaccines are for bacterial kidney disease (Renogen™), enteric

septicemia of catfish (AQUAVAC-ESC™) and columnaris disease (AQUAVAC-COL™). Safety studies required by the USDA Animal Plant Health Inspection Service, Center for Veterinary Biologics for these vaccines include using ten times the immunizing dose and direct fish to fish passage. In addition studies are done that consider the release of the vaccine into the environment and the ability of the vaccine to infect people (Shoemaker and Klesius, 2009).

DNA from DNA vaccines has a half-life ranging from three to six hours in fresh or marine aquatic environments. DNA attached to particulate material in an aquatic environment may last longer up to one hundred and forty hours (Lorenz and Wackernagel, 1994). Conditions required for bacterial transformation by the DNA are not likely to be found in open water (Alvarez et al., 1996). There is no evidence that naked DNA released from vaccination will enter into surrounding organisms.

Evaluation Question #5: Describe the toxicity and mode of action of the substance and of its breakdown products and any contaminants. Describe the persistence and areas of concentration in the environment of the substance and its breakdown products (7 U.S.C. § 6518 (m) (2)).

Vaccination of fish developed because the administration of antibiotics to fish in aquaculture was not sustainable: particularly for lower valued species (Midtlying, 1997). However, vaccination can lead to several types of toxic reactions for both the administrator and vaccinated animals. Self-injection on the fingers and hands of the operators can lead to allergic hypersensitivity and anaphylactic reaction. Improvements have been made with repeating syringes, such as the addition of a safety bow. However, adrenalin is recommended onsite, if self-injection does occur (Leira and Baalsrud, 1997).

Because food from fish is considered beneficial as a result of the effects of omega-3 polyunsaturated fats on cardiovascular and Alzheimer's diseases, more scrutiny has been given to the potential transmission of bovine spongiform encephalopathy from fish to humans. Serums used for the culture of viral vaccines and media used for the culture of bacterial vaccines may at times contain bovine products. Although the USDA, Animal Plant Health Inspection Service is not likely to permit licensed establishment to use contaminated products, prion free status should be verified for vaccines produced outside the jurisdiction of the USDA (Friedland et al., 2009).

Killed vaccines are inactivated with formaldehyde. During processing the vaccine is washed to remove formaldehyde, however; residual formaldehyde can produce toxic effects in fish. Furthermore, fish must be anesthetized prior to vaccination by injection. Stress caused by anesthesia is significant and likely adds to the potentially toxic effects of residual inactivating chemicals.

Some reports have described autoimmune disease development in farmed salmon after vaccination with oil adjuvanted vaccines. Granulomas are sometimes observed at the injection site. In addition fish can exhibit decreased carcass quality, spinal deformities, uveitis, and inflammation in the abdominal cavity (Haugarvol et al., 2010). There is a possibility of increased risk of infection with unvaccinated pathogens as a result of vaccine induced autoimmunity. On the other hand, vaccines that are adjuvanted with aluminum salts produce injection site lesions with much lower frequency. Notwithstanding, immunoprophylaxis can largely reduce risks for large scale animal suffering caused by disease epizootics in fish farming (Midtlying, 1997).

Evaluation Question #6: Describe any environmental contamination that could result from the petitioned substance's manufacture, use, misuse, or disposal (7 U.S.C. § 6518 (m) (3)).

Several studies have investigated a hypothetical set of unforeseen outcomes affecting the environment involving the administration of DNA vaccines (Gillund et al., 2008a, b). For example, 1) plasmid DNA (pDNA) remains in circulation in the aquatic system and is taken up by microorganisms that change as a result of the new DNA; 2) pDNA integrates into gonadial tissue of vaccinated fish resulting in offspring with disease resistance; 3) possibility for detection of pDNA in humans after they eat vaccinated fish; and 4) immunological consequences affecting these humans as a result of consuming the fish. Although not supported by strong evidence, these authors provide their information on the basis of the Walker and Harremoës (W&H) uncertainty framework, a tool to systematically identify scientific uncertainty. They

conclude that more research into the disposition of DNA vaccines for aquaculture into the environment needs to be done. Some information concerning the rareness of pDNA integration in marine bacteria mammals is available, but no information concerning actual adverse occurrences could be found (Lorentz and Wackernagel, 1994). DNA integration is a very rare event in vertebrates, even when DNA is deliberately introduced for the purpose of integration. Exhaustive studies have shown that DNA integration is possible, but the rate of integration is far lower than the rate of spontaneous mutation (Hepell and Davis, 2000).

In the cases of killed and modified live vaccines, there is a potential for incomplete inactivation for a particular vaccine lot leaving live pathogen in the vaccine and the reversion to virulence of the modified live vaccine inadvertently precipitating a new epizootic through vaccination. The vaccines themselves contain mostly organic material that rapidly degrades in the environment.

Vaccines produced under USDA license are manufactured in ultraclean manufacturing facilities. Both environmental and cross contamination are routinely avoided in these establishments and the solid wastes arising from them are scrupulously decontaminated using fumigation, or heat sterilization. Disposal of decontaminated material may be complicated by the addition of the decontaminants which include formaldehyde. The EPA provides stringent regulations for the discharge of this material. Thus, it is unlikely that vaccines for use in aquaculture produce environmentally detrimental waste as a result of their manufacture (OIE, 1991).

Evaluation Question #7: Describe any known chemical interactions between the petitioned substance and other substances used in organic crop or livestock production or handling. Describe any environmental or human health effects from these chemical interactions (7 U.S.C. § 6518 (m) (1)).

Most vaccines for aquaculture are manufactured directly from pathogens, some of which are pathogenic for humans as well. Bacteria are the main fish-borne zoonotic agents including: mycobacterium, *Streptococcus iniae*, *Erysipelothrix rhusiopathiae*, *Aeromonas* spp., *Vibrio* spp., *Edwardsiella* spp., *Salmonella* spp. and others. Even with vaccination there is always the risk of exposure to the pathogens themselves, including the potential of producing contaminated fish (Austin, 2010; Boylan, 2011). Prudent veterinary practice in aquaculture suggests vaccination of only healthy animals. This is not only to prevent needless suffering of animals, but also to prevent contamination of food with potentially zoonotic pathogens.

Evaluation Question #8: Describe any effects of the petitioned substance on biological or chemical interactions in the agro-ecosystem, including physiological effects on soil organisms (including the salt index and solubility of the soil), crops, and livestock (7 U.S.C. § 6518 (m) (5)).

The fish's immune system protects against diseases by detecting, identifying and removing pathogens; preventing the emergence of tumors and contributing to the processes that maintain stable conditions (homeostasis) during development and growth and after inflammatory reactions or tissue damage. The immune system is classically divided into the innate and the adaptive arms. The adaptive component of the teleost fish immune system drives the production of antibodies and cellular immunity in fish. The innate system is an evolutionarily ancient system present in both invertebrates and vertebrates (Magnadottir, 2010). The innate component of the fish immune system specifically recognizes pathogenic and non-pathogenic microorganisms and a number of molecules including DNA, RNA, cytokines, chemokines, interferon, polysaccharides, peptidoglycans, proteins, etc., with a set of inheritable germ line encoded pathogen pattern recognition receptors (PRRs) that initiate a response. These receptors sense particular structures in microorganisms called pathogen associated molecular patterns (PAMPs) representing literally thousands of specific molecules. The PAMPs and the PRRs mediate interactions with pathogenic and non-pathogenic microorganisms requiring coordination between multiple PRR signaling pathways that dictate the outcome of viral infection, and microbial colonization regardless of whether it is symbiotic coexistence, asymptomatic infection, or virulent disease (Boltana et al., 2011). The innate and adaptive immune receptors are functionally integrated into a single immune system that monitors the fish's immune health (Cohen, 2007). Many experiments have shown that fish, which survive infection, will show enhanced disease resistance or complete immunity on second encounter. A key element, as mentioned above, is adaptive immunity, the appearance of memory cells and specific antibodies. The basic aim of vaccination is

to imitate this process. Vaccination should thus activate both the innate and the adaptive system and lead to lasting protection (Magnadottir, 2010). Most of the current vaccines for fish contain adjuvants that stimulate the innate arm of the immune system and subsequently the adaptive arm. They do this by imitating PAMPs, increasing the innate response and augmenting the activities of the adaptive arm's cellular components such as dendritic cells, lymphocytes and macrophages, thus mimicking a natural infection. In some cases, this boost is very traumatic and results in extensive cell damage and potential immune disease (Israeli, 2009). Pathological changes can occur in various organs in farmed fish as a part of systemic autoimmune inflammatory conditions induced by vaccination. The vaccination protects the fish from a series of pathogens, but as a consequence, serious immune-related pathological conditions may be induced (Haugervoll et al., 2010).

One DNA vaccine, anticipated to be licensed in the US is available for infectious hematopoietic necrosis virus (Tonheim, 2008; APHIS, 2013c). DNA vaccines are injected into the muscle tissue of fish. DNA is a PRR that activates the innate immune system. Protein expressed from the DNA vaccine by phagocytic cells (macrophages, dendritic cells and lymphocytes) and at the injection site is presented by antigen presentation cells subsequently activating cells involved in the adaptive response: antibody production and cellular immunity. Residual DNA is mostly digested; however, DNA may be observed in the recipient cells up to 45 days post injection. Integration of pDNA injected intramuscularly in mice was studied with a sensitive polymerase chain reaction method showing no evidence of integration at a sensitivity of 1.3×10^{-9} integrations per cell (Ledwith et al., 2001). DNA vaccine integration in fish has not been established experimentally.

Naked DNA is neither infectious nor viable. Although free DNA is present in the environment it is not persistent. Data shows that extracellular DNA turns over rapidly in an aquatic environment (Alvarez et al., 1996). Natural genetic transformation of bacteria encompasses the active uptake by a cell of free extracellular DNA and heritable incorporation of its genetic information. Natural transformation only occurs in bacterial species (Lorenz and Wackernagel, 1994).

Since live vaccine strains (attenuated by natural selection or laboratory methods) are potentially released into the environment by vaccinates, safety issues concerning the veterinary as well as environmental aspects must be considered. These involve (i) changes in cell, tissue and host tropism, (ii) virulence of the carrier through the incorporation of foreign genes, (iii) reversion to virulence by acquisition of complementation genes, (iv) exchange of genetic information with other vaccine or wild-type strains of the carrier organism and (v) spread of undesired genes such as antibiotic resistance genes. Before live vaccines are applied, the safety issues must be thoroughly evaluated case-by-case. Safety assessment includes knowledge of the precise function and genetic location of the genes to be mutated, their genetic stability, potential reversion mechanisms, possible recombination events with dormant genes, gene transfer to other organisms as well as gene acquisition from other organisms by phage transduction, transposition or plasmid transfer and cis- or trans-complementation (Frey, 2007).

Evaluation Question #9: Discuss and summarize findings on whether the use of the petitioned substance may be harmful to the environment (7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i)).

The fish immune system is the first immune system in vertebrate evolution to possess an adaptive immune system. It enables fish to mount a unique lasting immune response against pathogens. Vaccines, through the adaptive immune system, permit fish to develop resistance to a pathogen in the absence of a virulent challenge.

Host density plays a role in the spread of fish diseases in the environment amongst farmed and wild fish. Low host density reduces the rate of encounter between susceptible hosts and pathogen. Increased host density will favor more rapid disease spread. In any population, there is a density threshold where disease spread can become epizootic. Effective aquaculture increases host density. Vaccines are effective at reducing the density of susceptible hosts and their use can lead to disease eradication. However, vaccination can be imperfect and lead to virulence evolution, potentially affecting wild and farmed fish and other species (Krkosek, 2010).

Evaluation Question #10: Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i)) and 7 U.S.C. § 6518 (m) (4)).

Self-injection appears to be the most important human health risk associated with aquaculture vaccination (Liera and Baalsrud, 1997). Otherwise, no adverse reports of zoonotic transmission to food resulting from vaccination have been reported. All vaccines used in the US or administered to animals in the US must be licensed by the US Department of Agriculture. In order for them to be licensed, they must be unequivocally shown to be safe for human health (APHIS, 2013).

Where a pathogen is infectious for both fish and humans, vaccinating fish can also prevent pathogen transmission to humans. Streptococcus iniae, a pathogen in tilapia, catfish, and striped bass can potentially cause cellulitis of the human hand. Vibrio and Edwardsiella both of which are fish pathogens may cause gastroenteritis and wound infection in humans (Austin, 2010; Boylan, 2011).

Evaluation Question #11: Describe all natural (non-synthetic) substances or products which may be used in place of a petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (ii)). Provide a list of allowed substances that may be used in place of the petitioned substance (7 U.S.C. § 6518 (m) (6)).

Farmed fish are kept at high population densities. In closed systems, where fish are raised in ponds or tanks, new fish stocks are introduced at various stages of development originating from domestic or international sources. While in open systems, fish are often in close proximity to wild fish reservoirs. These situations are ideal for the emergence of wild-type pathogens that exist benignly when fish are kept at low density. High host density increases the spread of aquatic pathogens between farmed fish and from farmed fish to wild fish that enter into or come into close proximity with net cages and with fish escaping from them (Kibenge et al., 2012).

The mucosal layer is the first line of defense against pathogens for fish. In fact, mucus covers all external surfaces of the fish, gills, and all of the internal surfaces of the gut. Epithelial cells secrete mucus which forms a protective barrier. The mucus layer contains many cells and functional substances supporting both innate and adaptive immunity. Under normal conditions, the mucus layer resists the penetration of pathogen bacteria and viruses, hosting an active gut flora that supports fish health (Cain and Swan, 2011). Early in development, commensal and favorable bacteria in the gastrointestinal (GI) tract stimulate immune activities and localized morphological development. The GI micro-flora plays a role in maintaining effective functionality after the GI tract develops (Dimitroglou et al., 2011). Normal micro-flora confers many benefits to the intestinal physiology of the host including metabolism of nutrients and organic substrates, and the contribution of the phenomenon of colonization resistance. However, when this balance is upset, pathogens that arrive or that have already been present but in numbers too small to cause disease take the opportunity to multiply. Probiotic supplementation can assist in returning a disturbed micro-flora to its normal beneficial composition, and influence the fish immune response in different ways. They can increase the proportion of phagocytically active cells and induce the activation of complement receptor expression. They also can modulate the secretion of anti-inflammatory cytokines (Gomez and Balcazar, 2007). Fish feeds with additives that enhance the GI tract micro-flora are preventative and intervention strategies against aquatic pathogens, e.g., pre-(manno/fructo-oligosaccharides; MOS/FOS) and pro-biotics (lactobacillus/lactococcus and bifidobacterium), immunostimulants (β-glucans, chitin, lactoferrin, levamisole) and nucleotides (Bacterial DNA containing methylated, CpGs) (Kibenge et al., 2012).

A number of herbal immunostimulants administered at various concentrations orally or through injection have been found to stimulate the innate and adaptive immune response in freshwater and marine fish against various bacterial, viral, and parasitic diseases. Herbal extracts can be used alone or with vaccines to enhance efficacy. Active substances in herbs include metabolic enhancers, immune system stimulants, broad spectrum antimicrobial and environmental stress relief (Harakrishnan et al., 2011). In one example, farmed kelp grouper, *Epinephelus bruneus* fed a diet enriched with a chaga mushroom (*Inonotus obliquus*) extract, protected against a virulent vibriosis challenge (Harkrishnan et al., 2012).

Evaluation Question #12: Describe any alternative practices that would make the use of the petitioned substance unnecessary (7 U.S.C. § 6518 (m) (6)).

Aquaculture diagnostic technologies are important not only to detect existing disease, but to predict disease movement. Improved applied methodologies for immunodiagnostics, direct or indirect fluorescence antibody, enzyme-linked immunosorbent assay, immune-chromatography and conventional nucleic acid-based approaches such as in situ hybridization using pathogen-specific gene probes, polymerase chain reaction (PCR), reverse transcription-PCR and quantitative real-time PCR (qPCR) can augment epidemiological models providing better information for the process of infection and progressive disease. Better models will allow greater control over movement of stock and placement of enclosures to limit infection and eradicate diseases. Recruitment of trans national organizations such as the International Organization for Animal Disease Control (OIE) and the World Health Organization (WTO) to assist in disease surveillance could improve resources for reducing the risk of international spread of aquatic animal diseases, including early warning of disease outbreaks, planning and monitoring of disease control programs, provision of sound aquatic animal health advice to farms, certification of exports, as well as international reporting and verification of freedom from particular diseases. The development of healthy and/or specific pathogen free stocks, genetic improvement of fish stocks (e.g., disease tolerant, growth rate and feed conversion efficiency) and documented histories that assure freedom from disease over will also facilitate reducing vaccine use (Browdy et al., 2012). Lower fish densities, good husbandry and attention to biosecurity in closed systems also support fish health.

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