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Pathogenic Organisms and Disease Prevention

Proceedings of the Twenty-ninth
U. S. -Japan Meeting on Aquaculture
Ise, Japan
November 7 and 8, 2000

Yasuaki Nakamura, Minoru Sorimachi, Tomoyoshi Yoshinaga,
James P. McVey, Paul Kilho Park and B. Jane Keller (editors)

Under the U.S.-Japan Cooperative Program in Natural Resources (UJNR)

National Research Institute of Aquaculture
Fisheries Agency

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Yasuaki Nakamura, Japan
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PREFACE

The United States and Japanese counterpart panels on aquaculture were formed in 1969 under the United States-Japan Cooperative Program in Natural Resources (UJNR). The panels currently include specialists drawn from government and academic departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture which could be of benefit to both countries.

The UJNR was begun during the Third Cabinet-Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, current subjects in the program include desalination of seawater, toxic microorganisms, air pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development and utilization.

Accomplishments include : increased communication and cooperation among technical specialists; exchanges of information, data, and research findings; annual meetings of the panels, a policy-coordinative body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects of international relations.

Yasuaki Nakamura - Japan
James P. McVey - United States

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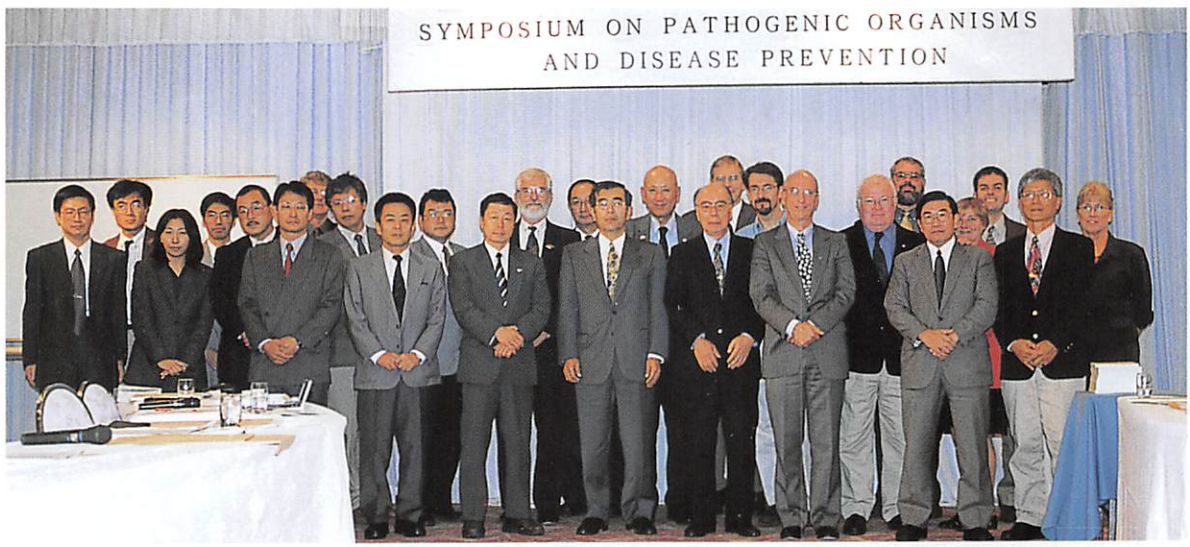

Participants is the 29th UJNR Aquaculture Panel Meeting

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ESTABLISHMENT OF THE LAW TO ENSURE SUSTAINABLE AQUACULTURE PRODUCTION

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ABSTRACT

In 1999, the Law to Ensure Sustainable Aquaculture Production was established as a legal framework to promote sustainable aquaculture in Japan. In this law, two issues are identified as major factors which may obstruct the establishment of a sustainable aquaculture production system. One is the problem of deterioration in the environmental condition of aquaculture ground caused by aquaculture itself, and the other is that of damage caused by infectious diseases of cultured aquatic animals and plants, especially by serious exotic diseases that have not occurred in Japan. The law consists of two major elements, the system of aquaculture ground improvement program and the measures to prevent spread of specific diseases (i.e. designated infectious diseases of aquatic animals and plants subjected to aquaculture, whose occurrence has not been confirmed, or only locally confirmed in Japan, and which could inflict serious damage to cultured aquatic animals and plants when spread). The former is the framework to promote voluntary and autonomous tackling of the problem of deterioration in the environmental condition of aquaculture grounds by fishery cooperative associations which have a license to engage in aquaculture. The latter is the regulations pertaining to occurrence of the specific diseases, such as the orders of prefectural governors to limit or prohibit transfer of the cultured aquatic animals and plants.

Key words: the Law, Sustainable Aquaculture Production, framework, Japan

INTRODUCTION

Japan has succeeded in increasing its aquaculture production, supported by development of production techniques and the economic growth. The aquaculture production in 1998 is 1.3 million metric tons, and there are some species almost all the production of which is putted out from aquaculture (Stastistics and Information Dept., MAFF, 1998). In addition, aquaculture has been contributing to the realization of affluent diet in Japan, since it has been producing mainly species which can be high-grade food, such as yellowtail or red sea bream. However, aquaculture in Japan is now coming up against many problems; deterioration in the environmental condition of aquaculture grounds caused by overcrowded aquaculture, damage from fish diseases, a drop of fish price due to overproduction or a business recession. Consequently, the production has reached the ceiling since the end of 1980's.

OBSTACLES TO THE ESTABLISHMENT OF A SUSTAINABLE AQUACULTURE PRODUCTION SYSTEM

In the report of the Committee on Fisheries of FAO in March 1995, it is mentioned that increased production from aquaculture would be essential if per capita consumption of fish were to be maintained at the present level. Needless to say, there is no doubt that aquaculture is quite important for future global food security, however, if we aim at enlargement of aquaculture production without appropriate management, it will be a menace to sustainability of the production. It is necessary to establish sustainable aquaculture production system, in order that aquaculture will eternally fulfill its responsibility. Actions to develop sustainable aquaculture are urgently required, as mentioned in Rome Consensus on World Fisheries/ adopted at the Ministerial Conference on Fisheries held just after the committee meeting.

In considering legal framework to promote sustainable aquaculture, Fisheries Agency of Japan (FAJ) identified

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two issues as major factors which may obstruct the establishment of sustainable aquaculture production system. One is the problem of deterioration in the environmental condition of aquaculture grounds caused by aquaculture itself, and the other is that of damage caused by infectious diseases of cultured aquatic animals and plants, especially, by serious exotic diseases that have not occurred in Japan.

In a sense, aquaculture grounds are resources which we can utilize eternally if we can manage them appropriately, conversely, we can not if we don't. It is just like that fishery resources are renewable and exhaustible natural resources, which can be depleted without proper management. Deterioration of aquaculture grounds brings about poor growth or death of cultured organisms from red tide or oxygen starvation, and can be a cause of outbreaks and spread of fish diseases. Furthermore, if we leave it as it goes, aquaculture in some grounds will be impossible to be continued. Therefore, in order to realize sustainable aquaculture, it is indispensable to keep the grounds in a good condition. However, as mentioned previously, there are regrettably not a few aquaculture grounds water quality and bottom environment of which is in a bad condition in Japan. In many cases, such bad condition is caused by accumulation of feed remnants or excrement from overcrowded aquaculture.

On the other hand, in recent years, fish diseases occur more constantly than ever, and proportion of incurable disease is getting larger. Consequently, damage from fish diseases has been reached 22-29 billion yen a year, which means 5-10% of total production in value from feeding aquaculture, although it fluctuates year to year (Estimation by Fisheries Agency based on questionnaire). In addition, nowadays, a risk of introduction of exotic fish diseases is getting bigger, as the import of fish seed is increasing.

THE LAW TO ENSURE SUSTAINABLE AQUACULTURE PRODUCTION

The Law to Ensure Sustainable aquaculture Production, which was drafted by Fisheries Agency of Japan under the recognition explained earlier, was established on May 21, 1999. This legislation is in line with the Kyoto Declaration adopted at the International Conference on the Sustainable Contribution of Fisheries to Food Security, which requests establishment of appropriate institutional and legal frameworks to promote sustainable and environmentally sound aquaculture.

The Law consists of two major elements, system of "Aquaculture Ground Improvement Program" and the measures to prevent spread of "the Specific Diseases (These are designated infectious diseases of aquatic animals and plants subjected to aquaculture, whose occurrence has not been confirmed, or only locally confirmed in Japan, and, which could inflict serious damage on cultured animals and plants when spread.)". Provisions relevant to the former became effective on the day of the establishment, the latter did on November 19, 1999.

THE BASIC GUIDELINE TO ENSURE SUSTAINABLE AQUACULTURE PRODUCTION

As a fundamental guiding principle to put the Law into practice, the Minister of Agriculture, Forestry and Fisheries shall establish "the Basic Guideline to Ensure Sustainable Aquaculture Production". The Basic Guideline was established on August 30, 1999.

It prescribes the matters relevant to, for instance, a goal of aquaculture ground improvement, measures for aquaculture ground improvement, preparation of organizational system for aquaculture ground improvement and prevention of spread of the specific diseases, and so on. As a matter of course, measures for aquaculture ground improvement include avoidance of overcrowded aquaculture (in other words, setting net pens of appropriate number and scale according to the aquaculture ground situation) , proper using of feed, appropriate management of aquaculture facilities, general measures for prevention of fish diseases, so as to reduce the loss by fish diseases.

Among these, matters relevant to a goal of improvement are to be a guideline for fisheries cooperative associations in setting up an improvement goal in their Aquaculture Ground Improvement Program.

AQUACULTURE GROUND IMPROVEMENT PROGRAM

In Japan, aquaculture is basically managed under a limited entry system based on the Fisheries Law, which is the basis of legal framework for fisheries regulation. In order to carry on aquaculture in public waters, it is necessary to get license of demarcated fishery right issued by a competent prefectural governor. Usually, license of demarcated fishery right is issued to fisheries cooperative associations, members of cooperatives engage in aquaculture respectively under the cooperatives' management (They set Exercise Regulation for Fishery Right as self-imposed control of cooperatives, which becomes effective when the governor authorizes it). Additionally, governments of prefectures where aquaculture is flourishing have guidelines for aquaculture on their own terms, and have instructed fish farmers to carry on aquaculture in an appropriate way.

In order to realize environmentally sound aquaculture, the Law introduced the system of Aquaculture Ground Improvement Program, complementing the existing management scheme as mensioned earlier. The Law

provides that cooperatives which have a license of demarcated fishery right can develop the Aquaculture Ground Improvement Program so that they can ensure sustainable aquaculture, and get approval of the governor.

Cooperatives are required to incorporate the contents of the approved program into their Exercise Regulation for Fishery Right, in order to make cooperatives' members be consistent with them.

As being understood from these, this system is legally based on voluntary (or self-imposed) activities of licensed cooperatives. The system is made to be not obligatory but voluntary, because it is concluded that deterioration of aquaculture grounds caused by aquaculture itself in Japan has not reached yet the level which needs legal controls at this moment.

The Law also provides a mechanism to make the system effective in practice, that is recommendation and other measures performed by the governor. In case a cooperative does not utilize its aquaculture ground in line with the Basic Guideline, and its aquaculture ground is recognized to be conspicuously deteriorated, the governor can recommend the cooperative to develop the Aquaculture Ground Improvement Program and/or to take other necessary measures. If the cooperative does not accept the recommendation yet, the governor can make it public. In case a cooperative does not make effort to improve its aquaculture ground in line with the approved program, the approval may be canceled.

MEASURES TO PREVENT SPREAD OF THE SPECIFIC DISEASES

In Japan, a legal framework to prevent the introduction of exotic fish diseases was established in 1996, with respect to the importation of aquatic animal seeds. Under the framework, those who intend to import seeds of designated aquatic animal species have to get permission of the Minister of Agriculture, Forestry and Fisheries. To get the permission, it is necessary to make an application accompanied by a certificate issued by a competent authority of exporting country attesting that the subject seeds of aquatic animals are confirmed or are believed not to be affected by infectious diseases (these are limited to the diseases prescribed by the Ministerial Ordinance). However, in case of introduction of serious exotic diseases, you need the effective system to prevent the diffusion of these diseases. Therefore, the Law introduced system to prevent diffusion of the Specific Diseases as a domestic prevention system of fish diseases. As the Specific Diseases, 9 diseases for 3 groups of aquatic animals have been already designated.

Those are;

- Spring Viremia of Carp for Cyprinids (Carp)
- Viral Haemorrhagic Septicaemia, Epizootic Haematopoietic Necrosis, Piscirickettsiosis, Enteric Redmouth for Salmonids,
- Epidemic caused by *Baculovirus penaei*, Epidemic caused by *Penaeus monodon* type Baculovirus, Yellow Head Disease, Infectious Hypodermal and Haematopoietic Necrosis for *Penaeus* (Prawn)

In case there is the possibility of the Specific Diseases' spread, the governor can make orders to owners or managers of cultured organisms which are infected or are suspected to be infected with the Specific Diseases.

These are;

- prohibition or restriction of movement of the cultured organisms
- incineration or burying of the cultured organisms
- sterilization of materials which are contaminated with pathogens of the Specific Diseases

Violations of these orders is to be subjected to penal regulations. Additionally, in order to prevent outbreak and spread of fish diseases including the Specific Diseases, the governor can make its officials to perform on-site inspection of aquaculture ground, and can request a necessary report from owners or managers of cultured organisms. The officials who perform the inspection are to be appointed as "fish disease prevention officials" by the governor. The governor can also ask those who have a lot of knowledge on fish disease from private sector to be "cooperators with the fish disease prevention officials" performing private activities on prevention of fish diseases.

POSTSCRIPT

Thus, a legal framework to promote sustainable aquaculture was established, focusing on problems of deterioration of aquaculture ground and damage from fish disease. However, this would be just one step to build sustainable aquaculture production system in Japan.

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HEALTH MANAGEMENT IN RED SNAPPER (*LUTJANUS CAMPECHANUS*) CULTURE

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ABSTRACT

The U.S. Gulf of Mexico Marine Stock Enhancement Program is using the red snapper to investigate the role of aquaculture technology as part of an overall management scheme. In our two-pronged approach, we assessed diseases and parasites through all life history stages of wild snapper. Then, we identified the parasites of wild fish that were potential risks to the culture system and minimized those risks through a comprehensive quarantine and monitoring program. Of over 35 species of parasites from wild specimens, some are direct threats to snapper health, some are direct threats to the culture system, and some are indirect threats, depending on conditions. Those from captive broodstock include an apparently harmless gall bladder myxosporean and two potentially destructive monogeneans. Some broodstock with equilibrium problems were infected with fungi that perforated the swim bladder and kidney. Several common bacteria infected snapper, but none is considered a primary disease agent. Cultured larvae have been free of infectious diseases, but cultured juveniles are susceptible to *Amyloodinium ocellatum*, a common parasitic dinoflagellate that can be controlled with careful administration of copper sulfate. Systemic granulomatosis and overall morbidity, perhaps due to an unidentified nutritional deficiency, were problems initially.

Key words: red snapper, aquaculture, *Lutjanus campechanus*, health management

INTRODUCTION

Worldwide, marine fish populations are declining. Some predict that the current capture fisheries, while relatively stable over the last 15 years, are unsustainable past the year 2040 (New, 1997). In the United States, population trends in those species for which they are known indicate that about two-thirds of the species are either at or below the levels required for sustainability (NMFS, 1999). In the Gulf of Mexico, at least five fish species, including the red snapper (*Lutjanus campechanus*) – the most popular foodfish in the Gulf, are listed as overfished by the National Marine Fisheries Service (NMFS, 1999).

The Magnuson-Stevens and Sustainable Fisheries Acts require that plans for management and restoration of overfished species be put in place. Stock enhancement, a combination of aquaculture and release of cultured fish into the wild, served as the technique of choice in marine fisheries management throughout most of the nineteenth and twentieth centuries. However, a century of enhancement activities produced little evidence of effectiveness (Blankenship and Leber, 1995). Further, concerns over maladaptive behaviors, artificial genetic selection, and disease problems in cultured fish created skepticism about the desirability of stock enhancement. In the face of growing concern over the continued decline of managed stocks, Blankenship and Leber (1995), citing studies from Japan (Tsukamoto *et al.*, 1989; Tsukamoto, 1993), Norway (Svasand *et al.*, 1990), and Hawaii (Leber, 1995), revived the idea that marine stock enhancement was possible through carefully planned research. Their paper outlined ten essential components of a "responsible" enhancement program that ultimately hinges on the ability to produce healthy juvenile fish for release.

The U.S. Gulf of Mexico Marine Stock Enhancement Program is patterned after the concept of Blankenship and Leber (1995). The program focuses on the issue of

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"responsible" stock enhancement by bringing together experts from a variety of fields, including ecology, behavior, genetics, management, production, and health first to address the concerns surrounding marine stock enhancement and second to integrate enhancement with traditional fisheries management techniques. The program is operated through a research consortium consisting of the Gulf Coast Research Laboratory (GCRL), Ocean Springs, MS; Mote Marine Laboratory, Sarasota, FL; and the Oceanic Institute, Waimanalo, HI. The consortium is using the red snapper as a model to test, refine, and demonstrate whether or not aquaculture technology can provide an additional tool to help restore overfished species. In this paper, we will present our program for health management that assures production of the healthy juveniles required for the red snapper enhancement program.

APPROACH

Diseases are an inherent part of the life histories of all organisms. Historically, the view popularized by Snieszko (1974) that the pathogen, the host, and the environment must interact under the proper circumstances to produce disease has determined our view of the disease process in fishes. Unfortunately, as Hedrick (1998) pointed out, this view may have caused us to focus too much on the "stress" of the culture environment as the facilitator of disease. Certainly, stress can produce disease, and the culture environment can produce stress; but the concept of disease must be broad enough to include ANY impairment that modifies normal functions (Wobeser, 1981). Thus, toxicants, climate, nutrition, infectious agents, and congenital defects may produce or interact to produce disease (Hedrick, 1998). Moreover, we must not forget that host organisms have evolved with (or perhaps because of) diseases (McIntyre and Amend, 1978; Ewald, 1994; Reno, 1998). Therefore, our understanding of the nature of disease must be tempered by our understanding of the life history of both the host organisms and the disease organisms.

Our program attempts to understand the nature of disease in the red snapper life history. The first step requires a thorough examination of all the diseases and parasites in all the life history stages of wild red snapper. From this information on wild fish, we can better identify diseases, parasites, or other factors that may represent potential risks in aquaculture and, as a result, design a system to minimize those risks.

Evaluation of aquaculture risks involves the realization that not all disease-causing agents are equal. Not every organism poses the same amount of risk to fish or culture systems. In general terms (at least with respect to disease-causing organisms), the potential risk is related to the life cycle of the parasite or disease organism. We have found approximately 35 species of parasites representing virtually every group of parasitic organisms in wild red snapper. These parasites can be categorized into three broad groups. Parasites that pose a direct threat to the health of red snapper constitute the first group. Parasites in this category are those that live in the tissues producing direct damage under normal circumstances and include digenean metacercariae, adult and juvenile acanthocephalans, and juvenile nematodes. Because these parasites require development in intermediate hosts, which are not generally present in culture systems, infections with these parasites are limited to wild fish (broodstock) entering the system. Parasites that pose a direct and significant risk to culture systems constitute the second group. Parasites in this category are those with direct life cycles that allow rapid multiplication/spread in a culture system and include monogeneans, leeches, and some copepods. The third group comprises those parasites that can, under the proper conditions, pose risk to culture systems. These parasites have a variety of life cycles and are mainly protoctists. Bacterial and viral agents also occur. These microbial agents could fit into any of the three categories above and can, of course, cause significant disease in culture systems; however, apart from one instance of a *Vibrio* infection in an otherwise debilitated fish, we have not seen evidence of microbial disease.

Non-infectious agents are more difficult to assess than the infectious ones. Our assessment of these relies primarily on trial-and-error experience in the aquaculture program. A large body of literature on non-infectious disorders of fish (e.g., Leatherland and Woo, 1998) as well as on fish nutrition (e.g., Halver, 1989), immunology (e.g., van Muiswinkel, 1995), and toxicology (e.g., Rand, 1995) provides a general framework. However, the particulars of how each fish species responds are unique. Therefore, given that the red snapper has never before been cultured in significant numbers, we must depend on trial-and-error experience while we develop the framework specific to this species.

AIM

One of the goals of responsible stock enhancement is to minimize disruption of the target ecosystem. Most theorists in the field of conservation biology consider such issues as habitat availability, trophic carrying capacity, and genetic diversity (Busack and Currens, 1995; Langton *et al.*, 1996; Pearsons and Hopley, 1999), but few (see McVicar, 1997) consider disease issues beyond the basic level. Currently, there is much debate concerning the importance of pathogen transfer between wild and cultured fish (see McVicar, 1997). Ultimately, all diseases of cultured fish originate from wild fish, and cultured fish have not been shown to introduce new diseases to wild fish;

however, naturally occurring diseases can be magnified or translocated. Therefore, the trend has been to discourage the release of fish with known diseases into populations of unknown disease status. Blankenship and Leber (1995) promote a broader view of disease issues in stock enhancement than most. Recognizing that disease is a natural occurrence, that we cannot control diseases in wild fish, and that the production of pathogen-free fish is impossible, they propose that released fish be allowed a level of infection equal to that in the target population.

Our program, therefore, aims to produce a product with a health status comparable to that of wild fish so that we can be confident that cultured fish pose little risk to wild fish and, conversely, that wild fish pose little risk to cultured fish. We adopt the ideas outlined by Lotz (1997) that call for high quality stock, multiple levels (streams) of production, risk identification, biosecurity, pathogen surveillance, and early warning as a means of disease control and quality assurance. We routinely administer a comprehensive inspection, sampling, and examination program that quickly and confidently detects potential problems.

THE PROGRAM

Our program consists of three steps. We harvest gametes from wild broodstock, produce larvae, and "grow out" the juveniles for release. The system is organized as three separate, completely independent components to compartmentalize the system and provide reduced risk to the system as a whole. Each component has its own inherent problems, requires its own routine standard procedures, and must be managed separately.

Broodstock

Because we currently depend on wild animals for broodstock, this facility incurs the most risk. Therefore, we segregate fish as they enter the facility according to purpose. Fish for immediate spawning are not placed directly into quarantine. They are grossly examined and then spawned in an isolated section of the facility. Recognizing that the link with wild fish is a potential problem in our health management system, we are developing two methods to alleviate the link with wild fish. We are developing a protocol for egg disinfection similar to that used in salmonid hatcheries. In addition, our long-term goal is to develop a genetically-managed, controlled maturation system that reduces the dependence on wild fish and gives us control over spawning.

Fish for maintenance and shipping purposes enter primary quarantine. Upon entry, we take a portion of the batch and perform a complete necropsy, which includes gross, histological, and microbiological examination, of virtually every organ system. The remainder of the batch is given a prophylactic treatment with Dylox® (o,o-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate) to rid the fish of the common ectoparasites that can endanger the facility. Fish are held in primary quarantine for 30 days during which time they are monitored for any abnormal signs or behaviors. Mortalities are completely investigated and the results of examinations are used in making decisions on further treatment. At the end of 30 days, a fish health statement that discloses known problems and treatments is produced. Fish can then be transported to our sister facilities where they enter the respective facility's secondary quarantine. We are developing a technique for using sentinel animals in the quarantine system. Periodic examination of these animals would, perhaps, allow earlier detection of agents or unexpected problems.

To date, we have relatively few problems in the broodstock facility. At least two species of monogeneans regularly enter the facility on wild fish, and we have seen evidence of their reproduction in the facility. The treatment with Dylox, which is intended to circumvent problems with ectoparasites, has, indeed, prevented these problems. We have identified several species of common bacteria that are normal components of the marine environment, but we have not noted any specific problem caused by the bacteria. One batch of fish contracted *Amyloodinium ocellatum* and suffered some mortality. Another batch of fish experienced an outbreak of *Cryptocaryon irritans* and was terminated. On one occasion, we identified two fungi *Penicillium corylophilum* and *Cladosporium sphaerospermum* perforating the swim bladder and kidney of two fish having difficulty maintaining equililbrium. The infections, however, were confined to the two individuals and were probably introduced through syringes used to evacuate the gases that expand when fish are pulled from significant depths.

Hatchery

Larvae are transferred into the hatchery at approximately 15 h post-hatch. Originally, larvae were produced at the Claude Peteet Mariculture Center, Gulf Shores, AL, and transported to GCRL; but, we now operate our own spawning program. When larvae are introduced into production, a representative sample of the larvae is taken for a general health evaluation and histological examination. Larvae are fed and maintained in the hatchery for approximately 30 days, during which time they are periodically sampled for health evaluation. We aim to sample at a level sufficient for confidently detecting any disease that is present.

Two main problems in the hatchery have been identified. One problem concerns the production of food. At present, we depend on nauplii produced from enhanced wild copepods to feed the larvae. Two issues arise from

this. First, because the degree to which we can produce copepod nauplii is dependent on the baseline abundance and composition of the wild copepod population, we can neither guarantee optimal levels of food nor ideal foods. Second, we cannot guarantee that diseases will not be introduced with the wild nauplii. Fortunately, we have found that red snapper feed readily on nauplii of a variety of copepod species. We, however, do not know how the varying nutrient composition of the different copepod species influences larval growth or survival. Studies on nutritional profiles of the various copepod nauplii and nutritional requirements of red snapper larvae are underway. With respect to the introduction of diseases, we attempt to minimize the risk by serial filtration through progressively finer sieves so that larvae receive a relatively pure size fraction of dietary components. Such fractionation plus a culture period of several days with no fish in the system has apparently kept infectious agents away from the nauplii. Closed system copepod culture would significantly reduce the risk of introducing diseases; however, the technology for mass culture of copepods in a closed system does not exist. We are currently attempting to develop that technology.

The second problem is higher than expected mortalities. The larvae are extremely sensitive to disturbance, which makes sampling for studies difficult. Most mortalities apparently occur before or during initiation of feeding. Indeed, mortalities associated with feeding initiation are not unusual in fish culture (reviewed by Speare, 1998). As a result, we believe these mortalities are not disease related. Rather, we suspect that larval quality plays a large role in this mortality event. A consistent, predictable, and, as yet, unexplained mortality event occurs at about day 19. Such an event may be programmed and, therefore, normal; but, it may also be related to nutritional deficiencies or developmental abnormalities. Work is underway to elucidate the cause of and, hopefully, minimize these mortalities.

Nursery

The nursery facility is housed in a location separate from the hatchery and is run on a closed, recirculating, treated water system. At about 30 days of age, the juveniles are transferred into the nursery. Before transfer, a health check is performed. In this manner, we ensure that the juveniles do not introduce anything into the clean nursery system. Routine sampling for health/histological examination continues every 10-14 days after transfer. Microbiological examination is performed as needed. Again, we aim to sample at a level sufficient for confidently detecting any diseases that are present.

Juveniles are generally robust. There are two significant health problems in the nursery. First, young juveniles display aggressive behavior toward one another. Larger fish typically kill and sometimes consume smaller fish resulting in as much as a 50% loss in the nursery. Research is underway to design an effective system or protocol to prevent this type of mortality.

The second health problem with the juveniles is the difficulty in preventing infestation with *Amyloodinium ocellatum*, a parasitic dinoflagellate to which the juveniles are extremely susceptible. Infestations by this organism rapidly become lethal. In two of the three years of this program, we have had some mortalities due to *Amyloodinium*. In neither of the two years could we definitively identify the source of the agent. In the first year, the problem probably arose from an emergency water exchange using improperly treated water. We do not know the origin of the problem in the second year, but we are confident that it did not originate from the hatchery because health screening did not detect the agent on the larvae. This year we prevented infection by restricting access to the facility, providing convenient disinfectant baths for equipment, and improving education of personnel in the various aspects of biosecurity.

Juveniles had some unexplained problems in the first year of the program. Fish were intolerant of handling and experienced chronic moribundity and systemic granulomatosis. Transmission experiments failed to indicate the presence of any infectious agent. There are at least three possible explanations for these problems. It is possible that we failed to adequately monitor copper sulfate levels during treatment for *Amyloodinium ocellatum* or that water quality deteriorated, resulting in unpredictable copper toxicity. We may not have provided proper nutrition to the fish resulting in one or several nutritional deficiencies. Finally, an interaction of the above conditions could have produced the observed effect.

Juveniles consistently display large fat deposits in both the body cavity and the liver. We are unsure as to the significance of the fatty livers because many species of fast-growing fish use the liver as a major fat storage organ (Roberts and Bullock, 1989). In addition, we have seen no evidence of negative effects from the fatty livers. Most fish probably lose the fatty deposits when they are released into the wild and develop "normal" feeding habits. Indeed, preliminary analyses of cultured fish recovered after 6 mo in the wild, indicate relatively normal livers. Research into the nutritional requirements of red snapper will, perhaps, result in the development of a more suitable pelleted food.

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ONCORHYNCHUS MASOU VIRUS (OMV) EPIDEMIOLOGY AND ITS CONTROL STRATEGY

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ABSTRACT

Distribution of salmonid herpesvirus was known in USA and Japan. Herpesviruses isolated in USA were classified to serotype 1 (SaHV-1) and in Japan were serotype 2 (SaHV-2). The reference strain of SaHV-1 is *Herpesvirus salmonis* and SaHV-2 is *Oncorhynchus masou* virus (OMV) strain OO-7812. OMV disease (OMVD) causes oncogenic and skin ulcer conditions. The main susceptible fish species are kokanee salmon (*Oncorhynchus nerka*), masu salmon (*O. masou*), coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*). Economic losses caused by this virus were recognized among kokanee salmon, coho salmon and rainbow trout. At the beginning of the 1980s, OMV was distributed widely in the northern part of Japan. Since 1988, OMV was isolated from coho salmon and since 1991 this virus was found in rainbow trout. Recently, re-appearing OMVD has become a major problem in pond culture of rainbow trout in the central part of Japan. OMV is sensitive to ultraviolet irradiation and iodophore treatment. Although detection of OMV in carrier fish is difficult using polymerase chain reaction (PCR) and OMV replicates and appears in ovarian fluid at mature stage, it can be controlled by disinfection. OMVD was successfully controlled by disinfection of all facilities and eggs with iodophore just after fertilization and again at early-eyed stage.

Key words: *Oncorhynchus masou* virus, OMVD, epidemiology, salmonid fish, herpesvirus, salmonid herpesvirus, SaHV-2

INTRODUCTION

A herpesvirus infection of salmonid fish in Japan was first described by Sano (1976) isolated from moribund kokanee salmon (*Oncorhynchus nerka*) in Towada Lake, northern part of Honshu, mainland of Japan. Subsequently, in 1978, a herpesvirus was isolated from the ovarian fluid of mature masu salmon (*O. masou*) cultured in Hokkaido. The virus was named *Oncorhynchus masou* virus (OMV) (Kimura *et al.*, 1981a). Following the discovery of OMV, many strains of herpesvirus that can be neutralized with antiserum against OMV have been isolated from cultured and wild salmonid fish in the northern part of Japan (Yoshimizu *et al.*, 1993). Since 1988, OMVD had become a major problem in pen cultures of coho salmon (*O. kisutch*) in the Tohoku district and since 1991 OMV has been found in pond cultures of rainbow trout (*O. mykiss*) in Hokkaido and the central part of Japan (Yoshimizu, 1996).

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NERKA VIRUS IN TOWADA LAKE (NeVTA)

High mortality has been observed among the fry of kokanee salmon (landlocked *O. nerka*) from June to September of every year since 1970. Mortality reached over 80% for the 3-mo period. The affected fish demonstrated the following signs: a darkening in body color, sluggish behavior and loss of appetite. From these moribund fish, a syncytium-forming virus was isolated in RTG-2 cells incubated at 10 ℃ in 1972 and 1974. The virus was classified as a member of Herpesviridae and it was named the nerka virus in Towada Lake, Akita and Aomori Prefectures (NeVTA) (Sano, 1976). NeVTA is pathogenic and lacks oncogenicity.

MASU SALMON HERPESVIRUS; *ONCORHYNCHUS MASOU* VIRUS (OMV)

In 1978, a herpesvirus was isolated from the ovarian

fluid of an apparently normal mature masu salmon (*O. masou*), cultured in the Otobe Salmon Hatchery in Hokkaido. This virus was named *Oncorhynchus masou* virus, after the scientific name of the host fish and its oncogenicity (Kimura *et al.*, 1981 a,b). The general properties of OMV were similar to those of *Herpesvirus salmonis* and NeVTA, but it differed in virion size and its optimal growth temperature. It was also distinct from *H. salmonis* with respect to its virus-induced polypeptide patterns and serological properties (Kimura and Yoshimizu, 1989). OMV was pathogenic and, more significantly, oncogenic for masu salmon and several other salmonid fish (Kimura *et al.*, 1981b). One-month-old kokanee salmon exhibited the greatest sensitivity. Masu and chum salmon also exhibited high susceptibility. Coho salmon and rainbow trout were shown to be less susceptible to OMV infection (Tanaka et. al., 1984). The incidence of tumor-bearing fish approached more than 60%. Epithelial tumors were found on 12-100% of the surviving chum, coho and masu salmon, and rainbow trout beginning at about 4 mo and persisting for at least 1 year post-infection (Yoshimizu *et al.*, 1987). Since its discovery in 1978 at the Otobe Salmon Hatchery, OMV has been isolated from the ovarian fluid and neoplastic tissue of mature masu salmon collected from other places. In 1981, a similar herpesvirus was isolated from the tissues of a basal cell carcinoma that developed on the mouthpart of yamame (another name for masu salmon) cultured at Koide Branch, Niigata Prefectural Inland Fisheries Experimental Station. This virus was named yamame tumor virus (YTV) (Sano *et al.*, 1983). Serologically, NeVTA, OMV and YTV were confirmed as being the same virus by Yoshimizu *et al.* (1995). Six representative OMV strains, which were isolated from ovarian fluid and tumor tissue of cultured as well as wild masu salmon in Hokkaido and Aomori Prefecture, were also confirmed as being the same virus by DNA restriction endonuclease cleavage analysis by Gou *et al.* (1991a). From the results of the comparison of the DNA homologies, OMV and YTV were classified as the same virus and NeVTA was classified as being similar yet distinct from these 2 viruses (Eaton *et al.*, 1991).

COHO SALMON HERPESVIRUS

Since 1988, herpesvirus has been isolated from the liver, kidney, and developing neoplasms in pond and pen-cultured coho salmon (Kimura and Yoshimizu, 1989; Kumagai *et al.*, 1994). Affected fish showed the following disease signs: skin ulcers, white spots on liver, and neoplastic tissues around mouthpart or body surface. Coho salmon culture was economically damaged by this disease. The herpesviruses isolated from coho salmon were tentatively named as coho salmon tumor virus (CSTV), *O. kisutch* virus (OKV) by Horiuchi *et al.* (1989), coho salmon tumor virus (COTV) by Kimura and Yoshimizu (1991) and coho salmon herpesvirus (CHV) by Kumagai *et al.* (1994). All of these viruses were neutralized by anti-OMV or NeVTA rabbit serum (Yoshimizu *et al.*, 1995), and the oncogenicity of CSTV, OKV and COTV was confirmed by artificial infection. In addition, restriction endonuclease profiles of CSTV were the same as those of NeVTA and YTV (Igari *et al.*, 1991). CHV showed strong pathogenicity to coho salmon (Kumagai *et al.*, 1994).

RAINBOW TROUT HERPESVIRUS

Since 1992, massive mortality has occurred among 1-year-old rainbow trout in pond cultures. The diseased fish exhibited almost no external signs. Some fish did manifest ulcerative skin lesions. Internally, intestinal hemorrhage and white spots on the liver were observed. No bacterial, fungal or parasitic agents were found and the herpesvirus was isolated from the kidney, liver, and ulcerative skin tissues. The rainbow trout culture industry experienced serious economic losses due to this disease, since rainbow trout of marketable size were affected and died. This herpesvirus was tentatively named rainbow trout kidney herpesvirus (RKV) by Suzuki (1993). The virus was neutralized with anti-OMV rabbit serum (Sung *et al.*, 1996) and its main characteristics were the same as OMV. RKV showed strong pathogenicity to marketable-size rainbow trout and masu salmon (Suzuki, 1993).

ROOTS OF OMV

Since 1978 to 2000, 25,753 females of 6 species of mature salmonid fish were collected to survey the incidence of this virus in Hokkaido and the northern part of Honshu. Herpesvirus was isolated from masu salmon at all the investigated sites with the exception of one hatchery. All of the isolates were neutralized with anti-OMV rabbit serum (Yoshimizu et. al., 1993). Based on our epizootiological and epidemiological study, the roots of OMV in Japan was assumed to be along the Japan Sea coast of Hokkaido and the presumed original host species was masu salmon. In the 1960s, eggs of masu salmon were collected from the rivers of the Japan Sea coast of Hokkaido, and transported to Honshu Island, mainland of Japan. With the unrestricted fish movement, the virus spread to several places in Honshu where the first cancer disease of masu salmon was observed (Kimura, 1976), and also in Hokkaido where OMV was already detected. Subsequently, coho salmon and rainbow trout were cultured in the same water systems where masu salmon was cultured. Coho salmon might be infected with OMV at fry stage in freshwater because when we found the tumor tissue around the mouth of pen cultured coho salmon, the

hatchery from where coho salmon was transplanted to pen had a history of OMVD (Kimura and Yoshimizu, 1991).

DIAGNOSIS

Detection of OMV in carrier fish is difficult but the virus replicates and appears in ovarian fluid at mature stage. For the purpose of virological survey of mature salmonid fish, ovarian fluid is collected by the method described by Yoshimizu at al. (1985), with the addition of an equivalent volume of antibiotic solution and reacted at 5℃, overnight. In the case of the tumor tissue, tissue is cut and disinfected with iodophore (50 ppm, 15 min), then washed with Hanks' balanced salt solution (BSS). Tumor tissue must be prepared for the primary culture or co-culture with RTG-2 cells. After one transplantation of primary culture cells, virus inspection of the culture medium should be carried out. In the laboratory, rabbit serum or monoclonal antibody against OMV was used for fluorescent antibody test (Hayashi *et al.*, 1993), and also polymerase chain reaction (PCR) and DNA probe were used for detection of unsymptomatic or diseased fish (Gou *et al.*, 1991b). PCR using a F10 primer, GTACCGAAACTCCGAGTC and R05 primer AACTTGAACTACTCC GGGG amplified a 439 base-pair segment of DNA from OMV strains isolated from masu salmon, coho salmon and rainbow trout, and liver, kidney, brain and nervous tissues. Agarose gel profile of amplified DNA was able to distinguish a SaHV-1 and SaHV-2. Sensitivity of this PCR was $10^{0.8}$ $TCID_{50}$/ml (Aso *et al.*, 2001).

CONTROL OF OMVD

OMV is sensitive to ultraviolet irradiation, ozone or iodophore treatment (Kimura and Yoshimizu, 1989). Since 1983, we have strongly recommended the inspection of the ovarian fluid from matured fish and the disinfection of collected eggs in almost all hatcheries in Hokkaido with iodine at the early-eyed stage as a control strategy. Currently OMV is no longer detected in most of the hatcheries in this area. Nowadays, all eggs and facilities have been disinfected by iodophore just after fertilization and again at the early-eyed stage. Formalin-killed OMV vaccine produced from OMV isolated from rainbow trout have been able to reduce the number of OMV replicating in ovarian fluid. As a result, OMV cannot be isolated in most of the hatcheries in this area, and could avoid the outbreak of OMVD (Yoshimizu *et al.*, 1993, 1995).

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DISEASES OF CULTURED MARINE FISH IN SUBTROPICAL AREAS OF JAPAN

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ABSTRACT

The marine fish aquaculture industry in Okinawa, constituting the subtropical part of Japan has been gradually developed, and mainly targets red sea bream *Pagrus major*, spangled emperor *Lethrinus nebulosus*, estuary cod *Epinephelus malabaricus*, and cobia *Rachycentron canadum*. Red sea bream iridoviral disease (RSIVD), edwardsiellosis, pseudotuberculosis, gliding bacterial disease, white spot disease, and skin fluke disease appear most often in fish cultured in Okinawa. Among these, RSIVD is the most serious in aquaculture. During seed production operations, gliding bacterial disease, epitheliocystis-like disease, and white spot disease can sometimes cause a large mortality rate among larvae and juveniles. In this paper, we review the status of major disease problems among marine fishes cultured in Okinawa.

Key words: viral diseases, bacterial diseases, parasitic diseases, subtropical Japan, marine fish, aquaculture

INTRODUCTION

The aquaculture industry in Okinawa Prefecture, constituting the subtropical part of Japan, has been gradually developed; total yield in value were 6.2 billion yen in 1998, accounting for 30.2% of the total annual fishery production in Okinawa (Figure 1). Main products are kuruma prawn, seaweed (*Cladosiphon okimuranus*), pearls, and red sea bream, corresponding respectively to 56.7%, 18.5%, 4.8% and 2.7% of the total production. Production based on marine fish aquaculture is still low, but has increased rapidly due to the development of successful seed production techniques for estuary cod, *Epinephelus malabaricus*. Since total fishery production has gradually decreased, marine fish aquaculture has become a promising fishery industry in Okinawa. Marine fish aquaculture in this area mainly includes red sea bream *Pagrus major*, spangled emperor *Lethrinus nebulosus*, estuary cod, and cobia *Rachycentron canadum*. Estuary cod and cobia are thought to be promising candidates for further promoting the aquaculture industry in this area.

RSIVD, edwardsiellosis, pseudotuberculosis, gliding bacterial disease, white spot disease, and skin fluke disease are the most common problems among cultured fish in Okinawa. In seed production operations, gliding bacterial disease, epitheliocystis-like disease, and white spot disease can sometimes cause a large mortality rate among larvae and juveniles.

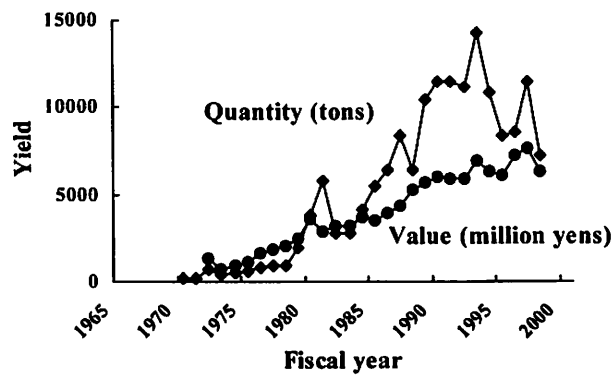

Figure 1. Aquaculture production of marine fish in Okinawa Prefecture.

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In this paper, we review the status of the major disease problems among marine fishes cultured in Okinawa.

PROFILES OF MAJOR FISH DISEASES IN OKINAWA

Red sea bream iridoviral disease (RSIVD)

The first outbreak of an iridoviral disease was reported in cultured red sea bream in Shikoku, Japan in 1990 (Inouye *et al.*, 1992). Many fish species show susceptibility to this disease which is caused by the infection of red sea bream iridovirus (RSIV) as reported by Matsuoka *et al.* (1996). RSIVD in red sea bream cultured in Okinawa Prefecture was first recorded in 1994, and mortality of cultured estuary cod has occurred since 1996. The disease is now very serious in this area, because there are no drugs which can be used in its treatment and because of the damage it causes to estuary cod, which is a promising candidate in aquaculture. RSIVD occurs during every season in Okinawa, especially from June to October or November, and it is frequently seen in cultured red sea bream and estuary cod. In contrast, severe losses due to RSIVD have not been observed in cultured spangled emperor and cobia. As a result of experimental infection, red sea bream and estuary cod have become highly sensitive to RSIV, but spangled emperor and cobia are less so, corresponding to the degree of disease occurrence in net cages. RSIV was transmitted through cohabitation experiments in estuary cod. Since this disease has not yet occurred in hatcheries in Okinawa, horizontal transmission of RSIV in cultured estuary cod seems to be the main route of infection as discussed by Nakajima *et al.* (1998).

Diseased estuary cod exhibits anemia of the gills and enlargement of the spleen. Histopathological examination shows the appearance of basophilic enlarged cells and diffused necrosis in the spleen and head kidney. RSIV has been routinely detected from diseased fish based on an immunofluorescent test using a monoclonal antibody (Nakajima *et al.*, 1995) and/or polymerase chain reaction (PCR) (Kurita *et al.*, 1998). Installing shelters such as beer bottle packing cases and rope coils in net cages seems to be effective in reducing stress in cultured estuary cod as well as mortality of fish affected by RSIVD.

Edwardsiellosis

The causative bacterium of this disease is reported as *Edwardsiella tarda* (Yasunaga *et al.*, 1982). Edwardsiellosis occurs occasionally in cultured red sea bream and Japanese flounder *Paralichthys olivaceus* in Okinawa. Japanese flounder, which is not distributed in this area, is mainly cultured on Iheya Island near Okinawa Island using cold seawater pumped from the underground. Affected red sea bream shows granuloma in the head and caudal peduncle. In diseased Japanese flounder, accumulations of ascites and hernia of the intestine are seen. The causative bacterium shows sensitivity to oxytetracycline, but once the disease occurs, it is difficult to completely treat.

Gliding bacterial disease

Flexibacter maritimus is a major causative bacterium of the gliding bacterial disease in cultured marine fish (Wakabayashi *et al.*, 1986). The causative bacterium, a gram-negative motile long rod, can be observed in smear samples of the lesions of affected fish in Okinawa, but its taxonomic status has not been confirmed. Affected fish frequently shows fin rot, ulcer formation on the body surface, and necrotic gills. Most cultured marine fish in Okinawa are sensitive to this disease in all seasons. In the hatchery, this disease causes mass mortality of larval and juvenile seeds in all fish species, especially groupers, estuary cod, and coral trout *Plectropomus leopardus*. Treatment with sodium nifurstyrenate is experimentally effective against this disease.

Pseudotuberculosis

This disease caused by the infection of *Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*) (Kusuda and Yamaoka, 1972) has occurred occasionally in cultured cobia, red sea bream and striped jack *Seriola dumerili*. In diseased fish, granuloma-like deposits can be seen as white spots in the spleen, liver, and kidney which are composed of a bacterial mass and an accumulation of phagocytes (Kubota *et al.*, 1970). In western Japan such as Kyushu and Shikoku, this disease usually ceases in midsummer at water temperature over approximately 27℃. However, this disease has frequently affected cultured cobia in every season, even in summer at water temperature over 30℃ in Okinawa since 1999. Administration of ampicillin is effective in treating this disease.

Lactococcus infection

Lactococcus garvie (formerly *Enterococcus seriolicida*) (Kusuda *et al.*, 1976) causes mass mortality of cultured yellowtail *Seriola quinqueradiata* in every season in Okinawa, similar to the situation of fish cultured in western Japan. Typical clinical signs are haemorrhage in the inside wall of

*1 Yuasa, K., Hussein, N. Kitancharoen, Y. Kataoka, S. Kawahara and K. Hatai (1997): Identification of *Streptococcus* isolated from cultured rabbitfish in the Persian Gulf and the efficacy of vaccination against the infection. Abstract. 4th Int. Symp. Japn. Soc. Fish Pathol., p. 109.

the operculum, ulcer in the caudal peduncle, exophthalmus, and haemorrhage in the liver and intestine (Kusuda *et al.*, 1976). Macrolide antibiotics such as erythromycin are effective in treating this disease.

Streptococcosis

Streptococcosis caused by *Streptococcus iniae* is a significant disease in cultured rabbitfishes such as *Siganus canaliculatus* and *S. guttatus* in Okinawa as reported in *S. fuscescens* in western Japan (Sugita, 1996) and in the countries along the Persian Gulf*[1]. Affected rabbitfish shows disparate swimming and disease symptoms are similar to those in infections of *L. garvieae*. The causative bacterium can be isolated from the brain, as well as from kidney and liver. Fish affected with *S. iniae* can be treated similarly as those infected with *L. garvieae*.

Epitheliocystis-like disease

This disease which causes mass mortalities of larvae in hatcheries was first reported in Kyushu (Egusa *et al.*, 1987); subsequently, this disease was observed in other hatcheries in Japan. Epitheliocystis-like bodies, which are histologically different from the epitheliocystis cells, form on the body surface, fins, spine, and gill branches of affected fish. Gram-negative bacteria can be seen when these bodies are squashed and smeared on a slide glass. Since the causative bacterium has never been isolated, its taxonomic position is not known. In hatcheries in Okinawa, larval seedlings of all fish species, especially groupers, estuary cod, and coral trout undergo severe damage. Once this disease occurs, almost all larvae die within a few days. In healthy juveniles of coral trout reared in a hatchery, these cyst-like bodies sometimes can be seen on the gills, but do not show any apparent impacts on the fish. Sodium nifurstyrenate may be effective in preventing infection by this bacterium.

White spot disease

This disease caused by the infection of a ciliate parasite *Cryptocaryon irritans* occurs in all cultured fish in Okinawa throughout the year. It severely damages fish cultured in shallow areas with poor water current or near estuaries. In particular, outbreak of this disease in net cages sometimes occurs under sudden environmental changes, such as decreased salinity due to heavy rainfall. This disease also causes frequent mass mortality of hatchery-reared larvae and juveniles in inland tanks.

Kudoa amamiensis infection

A great deal of cyst-like bodies were found in muscle tissue of yellowtail reared in the Okinawa International Ocean Exhibition at Motobu on Okinawa Island in 1975, and it was the first report of infection of the myxozoan parasite *Kudoa amamiensis* in Okinawa (Egusa and Nakajima, 1978). This disease is not lethal, but it reduces product quality. It has been reported to occur in Okinawa and Kagoshima (Amami-Ohshima) Prefectures. Egusa and Nakajima (1980) reported the presence of the same parasite found in the muscle of wild damselfishes. These fish were suspected to serve as natural hosts in this area and a source of infection in yellowtail, but experimental infection with this parasite from damselfish to yellowtail was not successful, and the source of the parasite or alternate host remains unknown. A recent epizootiological study conducted by Sugiyama *et al.* (1999) revealed that this disease occurs frequently in a limited area of Okinawa Prefecture. The prevalence of infection was over 70% in yellowtail cultured in this area from 1994 to 1998. The distribution of the alternate host, where transmission is necessary, may restrict the occurrence of the disease. New tools, including PCR and immunofluorescence test for the detection of the parasite at high sensitivity, were recently developed (Yokoyama *et al.*, 2000), and they are very useful not only for diagnosis but also epizootiological investigation of this parasite.

Skin fluke disease

Monogenean *Benedenia epinepheli* and *Neobenedenia girellae* infections have been reported to occur on the body surface and fins of many species of cultured marine fish (Ogawa *et al.*, 1995a, b; Koesharyani *et al.*, 1999), and *B. seriolae* has been observed on cultured yellowtail and amberjack (Ogawa and Yokoyama, 1998). In Okinawa, monogenean infections on the body surface of fish especially influence cultured yellowtail, amberjack, cobia, and estuary cod throughout the year. Species identity of the parasites has not been well examined. However, both *B. epinepheli* and *N. girellae* are suspected as causative parasites in cultured estuary cod, because these two monogeneans demonstrate very low host specificity, and several species of groupers *Epinephelus* spp. are known as hosts. Heavy infection of monogenean parasites causes fish mortality and, in addition, the presence of the infection easily induces secondary infections such as that of gliding bacteria. Immersing the affected fish in a freshwater bath is effective in removing these external parasites.

DISCUSSION

There already exist many diseases of fish cultured in Okinawa, which are similar to those in western Japan (see recent reviews: Muroga, 1995; Kusuda and Kawai, 1998; Nakajima *et al.*, 1998; Ogawa and Yokoyama, 1998). Fortunately, in hatcheries in Okinawa, there is no evidence

of the occurrence of viral nervous necrosis, which has been reported in hatchery-reared larvae and juveniles of many marine fishes including groupers (Yoshikoshi and Inouye, 1990).

In Okinawa, since water temperature exceeds 20℃ throughout the year and is higher than in western Japan, diseases can occur in all seasons. When new diseases invade this area, severe damage occurs to the aquaculture industry. Seeds of fish used in aquaculture have been introduced from abroad such as Taiwan and the Philippines which are near the Okinawa Islands. In Taiwan, Chou *et al.* (1998) reported an iridoviral disease of cultured grouper, which may differ from RSIV found in Japan. Special attention should be given to preventing the invasion of new pathogens carried by imported fishes, especially viruses, because there are no effective drugs which can be used for the treatment of viral diseases. In this respect, the Okinawa Prefectural Fisheries Experiment Station and the Okinawa Prefectural Sea Farming Center have expended great effort in developing technology for the mass seed production of economically important fish species in order to diminish the current reliance on the importation of fish from other countries. Collection of information concerning disease problems in other countries and detection methods of various pathogens are also essential in preventing disease invasion.

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ESTABLISHMENT OF A CULTURE SYSTEM FOR PISCINE NODAVIRUSES AND ITS APPLICATION TO VNN STUDY

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ABSTRACT

All genotypic variants of piscine nodavirus (the Betanodavirus), the causative agent of viral nervous necrosis (VNN) in larval and juvenile marine fish, were culturable in a fish cell line SSN-1. However, one problem in practical use of the SSN-1 cell line was that it is composed of a mixed population of cells, thus causing irregularity in cell constitution and inconsistency in the cytopathic effects (CPE) expression even if cultured under identical conditions. We overcame this problem by cloning SSN-1 cells, and a cell clone (E-11) obtained was highly useful for isolation and cultivation of all piscine nodaviruses due to its stable, clear CPE expression. Virus titration and growth experiments using the E-11 cell line clearly revealed differences in the optimal growth temperature among genotypic variants of piscine nodaviruses. The culture system we developed will be useful for future studies on piscine nodaviruses and VNN.

Key words: nodavirus, beta nodavirus, viral nervous necrosis, VNN, cell culture, SSN-1 cell line

INTRODUCTION

Piscine nodaviruses cause a highly destructive disease in hatchery-reared larvae and juveniles of a variety of marine fishes. Since first described in 1990, the disease named as viral nervous necrosis (VNN) has spread over the Indo-Pacific region, Mediterranean, and Scandinavia (Munday and Nakai 1997). Recently, the disease was recorded in North America (Curtis *et al.*, 2001). The affected fish exhibit a range of neurological abnormalities and are characterized by vacuolation and cell necrosis in the central nervous system and retina.

Nodaviruses have a bipartite genome of positive-sense RNAs, RNA1 encoding RNA-dependent RNA polymerase and RNA2 encoding coat protein, both of which are capped but not polyadenylated. The family Nodaviridae currently consists of two genera: the Alphanodavirus which primarily infect insects and the Betanodavirus which infect fish (Van Regenmortel *et al.*, 2000). Striped jack nervous necrosis virus (SJNNV), which had been purified from diseased larvae of striped jack *Pseudocaranx dentex*, was first identified as a member (type species) of the Betanodavirus (Mori *et al.*, 1992). The RNA1 (3.1 kb) and RNA2 (1.4 kb) of SJNNV encode 100 kDa (presumably RNA-dependent RNA polymerase) and a major coat protein of 42 kDa, respectively. Piscine nodaviruses are tentatively divided into four genotypes based on partial nucleotide sequences (about 430 bases) of the coat protein gene RNA2 (Nishizawa *et al.*, 1997), i.e., SJNNV, RGNNV (redspotted grouper nervous necrosis virus), TPNNV (tiger puffer nervous necrosis virus), and BFNNV (barfin flounder nervous necrosis virus).

Insect nodaviruses (the Alphanodavirus) can be propagated in a wide range of cultured cells and their RNA replication, gene expression and virion assembly have been well studied using these permissive cells (Ball and Johnson, 1998). In contrast, such studies on piscine nodaviruses have long been hampered by lack of a cell culture system. Early studies on isolation of piscine nodaviruses, in which some established fish cell lines such as RTG-2, CHSE-214, FHM, EPC, and BF-2 were used, reported unsuccessful results (Breuil *et al.*, 1991; Mori *et al.*, 1991; Munday *et al.*, 1992; Nguyen *et al.*, 1994; Grotmol *et al.*, 1995). The first successful isolation of a piscine nodavirus was made from diseased European seabass *Dicentrarchus labrax* using the SSN-1 cell line that had been established from whole fry

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tissue of striped snakehead *Ophicephalus striatus* (Frerichs *et al.*, 1996). Subsequently, Chi *et al.* (1999) reported that a new cell line (GF-1) derived from grouper *Epinephelus coioides* is useful for the isolation and proliferation of a piscine nodavirus (GNNV: grouper nervous necrosis virus). These permissive cell lines opened a new phase in virological and molecular biological studies on piscine nodaviruses.

This paper briefly reviews our recent results on the culture of piscine nodaviruses, and the applicability of culture systems to VNN studies is discussed.

SSN-1 CELL LINE

Seventeen isolates of piscine nodaviruses from larvae or juveniles of 13 marine fish species affected with VNN were examined for their infectivity to the SSN-1 cell line. These isolates were collected in 5 countries from 1991 to 1999 (Iwamoto *et al.*, 1999). They were inoculated in SSN-1 cells and incubated at 25℃. Based on cytopathic effects (CPE) and virus antigen detection by fluorescent antibody technique (FAT), the infectivity of the isolates were divided into 4 groups. Group I, including 9 virus isolates from 9 fish species, showed CPE characterized by rounded, granular cells with heavy cytoplasmic vacuoles within 3 d post-incubation (p.i.) and the monolayer partially or completely disintegrated over 3 to 6 d p.i. Group II, 3 virus isolates from striped jack, induced CPE with thin or rounded, granular, refractile cells without conspicuous vacuole formation and extensive FAT-positive reaction was observed in the time course similar to Group I. Cells inoculated with Group III (1 isolate from tiger puffer *Takifugu rubripes*) developed no distinct CPE but viral infection was evidenced by localized FAT-positive cells. There were no FAT-positive cells in Group IV including 4 isolates from 3 fish species. However, when incubation was performed at 20℃, the SSN-1 cells inoculated with the Group III isolate showed CPE similar to that of Group I and extensive FAT-positive reaction. Evidence of virus proliferation at 20℃ was also obtained in Group IV isolates. There is a good correlation between the infectivity to the SSN-1 cells and the coat protein gene genotypes of the isolates, i.e., Group I = RGNNV genotype, Group II = SJNNV genotype, Group III = TPNNV genotype, and Group IV = BFNNV genotype. These results indicate that SSN-1 cells are useful to propagate and differentiate all genotypic variants of piscine nodaviruses.

E-11 CELL LINE

However, the SSN-1 cell line has a disadvantage in its practical use; this cell line is composed of a mixed population of cells, causing inconsistencies in the CPE. In addition, the fact that the SSN-1 cell line is spontaneously infected by a C-type retrovirus designated as SnRV (Frerichs *et al.*, 1991; Hart *et al.*, 1996) may be another disadvantage in using this cell line. In order to overcome these problems, we cloned the SSN-1 cells and each cell clone was examined for permissiveness to piscine nodaviruses and the presence of SnRV (Iwamoto *et al.*, 2000). The results showed that 6 cell clones derived from the SSN-1 cell line were all susceptible to 4 piscine nodavirus strains belonging to different genotypes (SJNNV, RGNNV, TPNNV, and BFNNV). Three clones, designated as A-6, E-9, and E-11, were highly permissive to nodavirus infection and production. The virus-induced CPE appeared as cytoplasmic vacuoles and intensive disintegration at 3 to 5 days p.i. These observations were highly reproducible and formed the basis for a successful virus titration system. Quantitative analysis using the cloned E-11 cell line clearly revealed differences in the optimal growth temperatures among the genotypic variants: 25 to 30℃ for RGNNV, 20 to 25℃ for SJNNV, 20℃ for TPNNV, and 15 to 20℃ for BFNNV. Electron microscopy demonstrated SnRV retrovirus particles only in A-6 and E-9 cells, but PCR-amplification for *pol* gene encoding reverse transcriptase and LTR (long terminal repeats) region of the proviral DNA indicated the presence of the retrovirus in the other clones including E-11. Although SnRV-free permissive cells were not cloned, the clones obtained here, especially E-11, will be more useful for qualitative and quantitative analyses of piscine nodaviruses than the SSN-1 cell line.

RAPID AND SENSITIVE DETECTION OF THE VIRUS

The E-11 cells are highly sensitive to piscine nodaviruses but it takes a maximum of 10 days after inoculation to detect the virus at lower numbers based on the CPE expression. In contrast, the RT-PCR is generally a rapid and convenient method to examine a large number of samples but its sensitivity is not so high. The RT-PCR technique required 10^4 $TCID_{50}$ for detection of a strain of the SJNNV genotype or 10^5 $TCID_{50}$ for strains of the other 3 genotypes (RGNNV, TPNNV, BFNNV). Accordingly, we developed a procedure by combining advantages of both methods, i.e., high permissivity of E-11 cells and rapidity of RT-PCR (Iwamoto *et al.*, 2001). When the 72-h culture of virus in the cells was examined by the RT-PCR, a positive-PCR amplicon was obtained from every genetic variant sample containing virus particles at the lowest number (10^0 $TCID_{50}$). The cultivation for 24 h prior to RT-PCR was enough to detect the virus at the lowest titer. Therefore, this procedure, preculture in the E-11 cells and RT-PCR, will be useful as a rapid and sensitive method for detection of piscine nodaviruses.

FURTHER APPLICATION OF THE CULTURE ASSAY SYSTEM

In VNN of striped jack, virus-carrying broodstock were shown to be the most important inoculum source of the virus to their larvae (Arimoto *et al.*, 1992; Mushiake *et al.*, 1994). This finding led to successful control of VNN in larval striped jack, where elimination of virus-carrying broodstock by RT-PCR and disinfection of fertilized eggs by ozone were practiced (Mori *et al.*, 1998). However, it remains unclear whether this vertical transmission mode of the virus is common for all host fish species, though piscine nodaviruses also have been detected in the gonadal materials of broodstock of kelp grouper *E. moara* or European seabass (Nakai *et al.*, 1994; Comps *et al.*, 1996). For this reason, no control measures have been established in VNN of fishes except striped jack. The culture assay system we developed will reveal the pathogenesis of the disease to find a clue for disease prevention. Furthermore, with this culture system, we developed a virus-neutralizing assay system and demonstrated the presence of nodavirus-neutralizing antibodies in serum of the sevenband grouper *E. septemfasciatus* which survived intramuscular injection with the virus (Tanaka *et al.*, 2001). This antibody assay system will be useful for the future vaccine development of VNN. Needless to say, the culture system will open a gate for molecular analysis of piscine nodaviruses.

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RED SEA BREAM IRIDOVIRAL DISEASE IN JAPAN

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ABSTRACT

The first outbreak of red sea bream iridoviral disease (RSIVD) caused by red sea bream iridovirus (RSIV) was recorded among cultured red sea bream (*Pagrus major*) mainly among juveniles around Shikoku Island, Japan, in 1990. However, related mortalities of market-sized fish have also been reported. Since 1991, the disease has caused mass mortalities of cultured marine fish. It is known that the RSIVD occurred among 31 cultured marine fish species in the western part of Japan. The diseased fish were lethargic and showed severe anemia, petechiae of the gills, and enlargement of the spleen. The disease was histopathologically characterized by the appearance of enlarged cells that were deeply stained with Giemsa solution in the spleen, heart, kidney, liver and gills. An immunofluoresence test with a monoclonal antibody (MAb) and a polymerase chain reaction (PCR) assay for rapid diagnosis of the disease has been developed. The indirect immunofluorescence test with a MAb is commonly used for rapid diagnosis of RSIV-infected fish in the field. For effective control measures, a formalin-killed vaccine was developed and shows a significant effect in red sea bream under both experimental and field conditions. The vaccine was also effective in other cultured marine fish such as the yellowtail (*Seriola quinqueradiata*). The vaccine is now commercially available in Japan for red sea bream and yellowtail.

Key words: Viral disease, cultured marine fish, Iridovirus, vaccine, diagnosis

INTRODUCTION

The first outbreak of a red sea bream iridoviral disease (RSIVD) was recorded in cultured red sea bream (*Pagrus major*) in Shikoku Island, Japan, in 1990. Since 1991, the disease has produced mass mortalities of cultured fish populations in the western part of Japan, mainly among juvenile red sea bream. However, mortalities of market-sized fish have also been reported. The same type of disease has also seriously damaged stocks of several kinds of cultured marine fish such as yellowtail (*Seriola quinqueradiata*), sea bass (*Lateolabrax* sp.), and Japanese parrotfish (*Oplegnathus fasciatus*).

In this paper, we briefly review previous works on RSIVD and describe current studies on the control measures by vaccination.

CLINICAL PATHOLOGY

The affected fish were lethargic and exhibited severe anemia, petechiae of the gills, and enlargement of the spleen (Inouye *et al.*, 1992). The disease is characterized by the appearance of enlarged cells stained deeply with Giemsa solution in microscopic observation of tissue sections of the spleen, heart, kidney, liver, and gills of infected fish (Inouye *et al.*, 1992). The most typical histological change observed in affected fish is the appearance of enlarged cells in the spleen. Hexagonal virions were found in the cytoplasm of these cells and the viral particles form crystalline arrays.

CAUSATIVE VIRUS

It was reported that the causative agent was a large, icosahedral, cytoplasmic DNA virus classified as a member of the family Iridoviridae (Inouye *et al.*, 1992). The virus was designated as red sea bream iridovirus (RSIV). Each virion consists of a central electron-dense core (120 nm) and an electron-translucent zone, measuring 200-240 nm in diameter. Biological and physico-chemical properties of the virus have been reported (Nakajima and Sorimachi, 1994). RSIV is grown on GF, BF-2, CHSE-214, FHM, JSKG, KRE-3, RTG-2 and YTF cell lines. The titers of the virus on GF, BF-2 and KRE-3 are higher than those on

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the other cell lines. The pretreatment of BF-2 and KRE-3 cells with polyethylene glycol (PEG) or addition of PEG to the sample increased the virus titers. RSIV is replicated at 15℃, 20℃, 25℃ and 30℃ but not at 37℃. The suitable temperature for viral growth is 20℃ or 25℃. The virus is sensitive to acid (pH3), chloroform and ether and unstable to heat but stable to ultrasonic treatment and repeated freezing and thawing. Treatment with 5-iodo-2-deoxyuridine (IUdR) reduced the titer of the virus.

RSIV virions contain linear double-stranded DNA genomes. The complete nucleotide sequence of RSIV was determined. The genome of RSIV is about 112 thousand base pairs (bp) in length and contains about 90 potential genes (unpublished data).

The antigenic relationship between RSIV and two iridovirus-like agents associated with systemic infection in fish--epizootic haematopoietic necrosis virus (EHNV) and the iridovirus isolated from sheatfish (SFIV) -- were examined. Although cross reactivities were observed between RSIV and other fish iridoviruses by immunofluorescence test or immunoprecipitation test using anti-RSIV serum, none of the MAbs against RSIV reacted with EHNV or SFIV-infected cells by the immunofluorescence test (Nakajima *et al.*, 1998). Pathogenicities of EHNV or SFIV to red sea bream were not shown by experimental challenge (Nakajima and Maeno, 1998).

ETIOLOGY

It is known that the RSIVD has afflicted 20 species of cultured marine fish in 17 prefectures located in the southwestern part of Japan from 1991 to 1995. The infected fish include 18 Perciformes, 1 Pleuronectiformes and 1 Tetradontiformes (Matsuoka *et al.*, 1996). From 1991 to 2000, it is known that the RSIVD occurred among 31 cultured marine fish species which belong to Perciformes, Pleuronectiformes or Tetradontiformes in the western part of Japan (unpublished data). The infected fish include yellowtail, sea bass, Japanese parrotfish, amberjack (*Seriola dumerili*), goldstriped amberjack (*Seriola aureovittata*), striped jack (*Pseudocaranx dentex*), horse mackeral (*Trachurus japonicus*), albacore (*Thunnus thynnus*), Japanese flounder (*Paralichthys olivaceus*) and tiger puffer (*Takifugu rubripes*). The viral etiology was demonstrated by transmission experiments using a filtrate of homogenates from RSIVD (Inouye *et al.*, 1992). Intraperitoneal inoculation of the filtrate (450nm) of spleen homogenate of the infected fish to juvenile red sea bream induced similar pathological changes to those observed in spontaneously diseased fish, and the virus or viral antigens were detected from them. In addition, intraperitoneal inoculation of homogenate of RSIV-infected amberjack, striped jack and albacore to juvenile red sea bream induced similar pathological changes (Nakajima and Maeno, 1998). The infection was also established in healthy red sea bream by cohabitation or in water from the tanks rearing RSIV-infected fish (unpublished data). These results suggest a possibility of horizontal transmission of RSIVD. Until now, RSIVD has not occurred in hatcheries; thus, the possibility of vertical transmission seems to be little.

DIAGNOSIS

Diagnostic methods such as the observation of stamped or sectioned specimens stained with Giemsa, an immunofluorescence test with a MAb and a polymerase chain reaction (PCR) have been reported (Inouye *et al.*, 1992; Nakajima and Sorimachi, 1995; Nakajima *et al.*, 1995; Oshima *et al.*, 1996, 1998; Kurita *et al.*, 1998).

The diagnosis of RSIVD by an immunofluorescence test with a MAb is based on methods that are either the isolation of RSIV in cell culture followed by its identification with anti-RSIV M10 MAb or direct demonstration of antigens in infected tissue using M10 MAb. The indirect immunofluorescence test using a M10 MAb revealed that specific fluorescence was observed in imprints or frozen sections of spleens, not only in red sea bream but also in other fish (Nakajima *et al.*, 1995). Thus, the indirect immunofluorescence test with a MAb is commonly used for the rapid diagnosis of RSIV-infected fish in the field.

The localization of the reactive antigen with anti-RSIV polyclonal antibody and M10 MAb was examined by immunoelectron microscopy. With anti-RSIV rabbit antiserum, the reactive antigen was found in the virion. Although the reactive antigen was not found in the virion with M10 MAb, an antigen was detected on the surface of the virus-infected cells (unpublished data). These results suggest that the MAb recognizes a virus-induced polypeptide that was located on the surface of the virus-infected cells.

Based on the sequence data of RSIV, a PCR technique has been developed to detect RSIV. Four oligonucleotide primer sets based on the ATPase gene, DNA polymearse gene and a Pst I-restriction fragment of RSIV genomic DNA were synthesized to amplify the RSIV DNA of 563-570 bp length (Kurita *et al.*, 1998). Furthermore, since the target region among RSIV was successfully amplified from diseased fish other than red sea bream, PCR using primers designed for RSIV has a broad application for the diagnosis of RSIVD in a number of species (Kurita *et al.*, 1998).

CONTROL

The prevention and control of fish virus diseases are serious problems, especially among cultured fish. There

are no antibiotics or chemotherapeutic compounds which are effective virustats or virucides. Therefore, control methods for RSIVD currently rely on the implementation of hygiene practices at the farm. Recently, we have developed a formalin-killed vaccine which shows a significant effectiveness in red sea bream under both experimental and field conditions (Nakajima *et al.*, 1997, 1999). In experimental infection, juvenile red sea bream were intraperitoneally injected with the vaccine and after 10 days the fish were RSIV-challenged by intraperitoneal injection. Statistical analysis showed significantly high survival rates in the vaccinated groups than in those of the respective control groups. In a field trial, two groups each consisting of 1,000 juvenile red sea bream were either intraperitoneally inoculated with the vaccine or were not vaccinated. After vaccination, fish were transferred to marine net pens and observed for 12 wk. The cumulative mortalities caused by RSIVD in the vaccinated or control groups were 19.2 and 68.5%, respectively. The vaccine is now commercially available in Japan for red sea bream and yellowtail. However, further studies are needed on the route of administration of this vaccine and the effectiveness of the vaccine for cultured marine fishes other than red sea bream and yellowtail. Although the effectiveness of vaccination was shown, the most important prophylactic method is to reduce various stress factors on the fish, because the establishment of the infection generally depends on a balance between the amount of invading agent and the defense mechanisms of the host.

The sera obtained from experimentally and naturally RSIV-infected fish reacted with the 46 kDa polypeptide by radioimmunoprecipitation test (unpublished data). The sera obtained from vaccinated fish also reacted with the 46 kDa polypeptide. This shows the possibility that the antibody to this polypeptide is important in the humoral immunity against RSIVD.

DISCUSSION

This disease was first reported in 1990 and its spread has affected various kinds of cultured marine fishes in Japan. In addition to the results obtained through studies of RSIVD, the following investigations among RSIVD and RSIV will be required to understand this disease. The infection source of this disease is not clear. There is a possibility that the disease was imported from foreign countries with seedlings, but the confirmation has not been made. This virus is detected from diseased fish mainly in summer but not in winter time. The existence of the virus is not clear in winter; therefore, epidemiological studies are needed to clarify the ecology of the pathogen. RSIV isolated from various fish species in Japan are shown to be closely related to each other by reaction patterns against antibodies and virion polypeptide profiles (Nakajima *et al.*, 1998). In relation to the infection source, the comparison between RSIV and other iridoviruses isolated in foreign countries especially in southeast Asia where Japan has imported a lot of seedlings is needed.

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EFFICACY OF A DNA VACCINE AGAINST INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is a significant salmonid pathogen for which there is no commercially efficacious vaccine available. Candidate vaccines based on attenuated virus strains, inactivated virus, protein subunits, or peptides are not in current use due to concerns regarding cost, safety and/or efficacy. A DNA vaccine containing the glycoprotein gene of IHNV has been shown to stimulate a protective immune response in rainbow trout (*Oncorhynchus mykiss*) fry and immature adults when administered at very low doses. An optimal dose of 0.1 μg DNA, injected intramuscularly into 1 g fry, provides highly significant protection against a virulent virus challenge. In our continuing investigations of the efficacy of this vaccine, we have found that it protects rainbow trout fry against challenge with heterologous IHNV strains including an extremely virulent IHNV strain, 220-90, and other strains from North America, Japan, and France. In a comparison of six different vaccine delivery methods, both intramuscular injection and cutaneous particle bombardment using a gene-gun elicited significant ($p<0.05$), nearly complete protection. Intraperitoneal injection also provided significant ($p<0.05$) protection but with a much lower relative percent survival. Finally, a study of the duration of protection is in progress and indicates that significant ($p<0.05$) protection is still provided against viral challenge one year after vaccination, although the protection is not as strong as that observed at earlier timepoints. Together these data contribute toward our ultimate goal of developing an effective vaccine for protection of salmonids against IHNV disease.

Key words: IHNV, infectious hematopoietic necrosis, fish vaccine, DNA vaccine

INTRODUCTION

The most significant viral pathogen of salmonid fishes in North America is infectious hematopoietic necrosis virus (IHNV). This virus has a geographic range extending from Alaska to California and inland to Idaho, and it is endemic to nearly all watersheds in this range that have salmon or trout populations. Disease outbreaks of infectious hematopoietic necrosis (IHN) occur at numerous locations every year, resulting in substantial losses to both cultured and wild fish populations. Due to its economic importance, IHNV has been well characterized both biologically and at the molecular level (Wolf, 1988; LaPatra, 1998). IHNV is in the family *Rhabdoviridae*, and it is the type strain of the newly recognized genus, the novirhabdoviruses (Walker *et al.*, 2000). Virus particles of IHNV are covered with trimeric spikes of a homogeneous glycoprotein, the G protein, and it has been shown that this is the major antigenic protein that stimulates a protective immune response in susceptible salmonid hosts (Engelking and Leong, 1989).

Despite considerable research over the last 20 years to produce a vaccine against this virus, there is no commercial IHNV vaccine available today (Winton, 1997). Traditional vaccine formulations such as inactivated virus or attenuated IHNV strains are efficacious but they have not been commercialized due to cost or safety concerns. Molecular vaccines composed of IHNV protein subunits or peptides have had difficulties with efficacy or consistency, rendering them thus far unsuccessful. In 1993, Ulmer and his research group published the first report of a novel type of vaccine, a DNA vaccine. They described protection of

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mice against influenza virus after they were injected with a DNA plasmid containing the gene for an influenza virus surface protein. A DNA vaccine delivers to the host the gene for an antigenic protein of a pathogen, rather than delivering the protein itself. In many cases, this results in a better immune response than traditional vaccines, presumably because the antigenic protein is synthesized in the host *in situ* in a manner that more closely mimics its synthesis during a natural infection. The first report of a DNA vaccine for fish hosts was published by Anderson *et al.* (1996), showing protection of rainbow trout fry from IHNV after vaccination with 10 μg of a DNA vaccine containing the gene for the IHNV glycoprotein (G) protein. We have built on that early work by producing a second generation IHNV DNA vaccine (pIHNVw-G) in a new plasmid vector, and demonstrating that this vaccine also provides strong protective immunity in rainbow trout fry vaccinated at doses of 1-10 μg (Corbeil *et al.*, 1999). This paper describes further testing of this vaccine to characterize many important and exciting aspects of its efficacy *in vivo*.

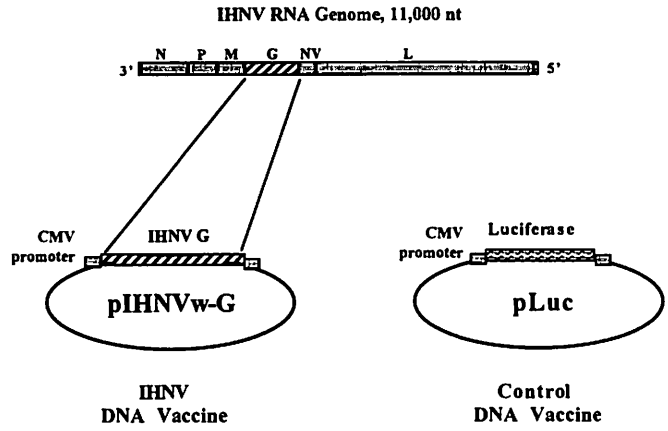

Figure 1. Structure of the pIHNVw-G DNA vaccine and the pLuc control DNA vaccine. The horizontal line at the top represents the linear IHNV genome with six genes. The pIHNVw-G DNA vaccine was constructed by inserting a cDNA copy of the full length glycoprotein (G) gene from the WRAC strain of IHNV into the circular plasmid vector pCDNA3.1 (In Vitrogen) . The control vaccine was made by inserting the luciferase marker gene into the same vector. Expression of the inserted genes is driven by the immediate-early, enhancer-promoter of the human cytomegalovirus (CMV).

MATERIALS AND METHODS

DNA Vaccines

The construction of the IHNV DNA vaccine, pIHNVw-G, has been described previously (Corbeil *et al.*, 2000b). This vaccine contains a cDNA copy of the glycoprotein (G) gene of the WRAC strain (also called 039-82, LaPatra *et al.*, 1994) of IHNV inserted into the pCDNA3.1 DNA vaccine vector (InVitrogen). The structure of this vaccine is shown in Figure 1, along with the structure of a control vaccine containing the luciferase gene rather than the IHNV G gene. The luciferase gene functions as a control and has no biological relevance to IHNV. Once delivered to the host, both the IHNV vaccine and the luciferase control vaccine express their respective proteins under the control of the eukaryotic cytomegalovirus (CMV) immediate-early promoter-enhancer. Both vaccine plasmids were amplified in *Escherichia coli* strain DH5a and plasmid DNA was purified by the alkaline lysis protocol of Saporito-Irwin *et al.* (1997).

Fish and challenge virus stocks

All experiments were conducted using the Clear Springs strain of rainbow trout (Clear Springs Foods Inc., Buhl, ID) at various sizes as indicated in the text. Fish were maintained in tanks receiving sand-filtered and UV-treated lake water at 15℃, and they were fed a standard dry pelleted diet daily. The challenge strain of IHNV for most experiments was either the WRAC strain or the more virulent 220-90 strain (LaPatra *et al.*, 1994). The study of protection against heterologous virus strains used 7 different challenge virus strains as described in Corbeil *et al.* (2000b). These included WRAC, 220-90, RB1 (Hsu *et al.*, 1986), Col85 (Nichol *et al.*, 1995), AK14 (Emmenegger *et al.*, 2000), Shizuoka (Winton *et al.*, 1988), and 32-87 (Arkush *et al.*, 1989). All challenge virus strains were propagated in epithelioma papulosum cyprini (EPC) or chinook salmon embryo (CHSE-214) cells and titered as described (Corbeil *et al.*, 2000b).

Vaccination

For standard vaccination, fish were anesthetized by immersion in 100 μg/ml of tricaine methane sulfonate (MS-222, Argent Chemical Laboratories) and injected intramuscularly at the base of the dorsal fin with the specified vaccine dose using a needle and syringe on a repeating pipettor. Vaccine doses ranged from 0.001 μg to 10 μg of plasmid as described in the text, and were delivered in 50-100 μl of phosphate buffered saline (PBS). In addition to pIHNVw-G and pLuc vaccinated fish, additional control groups included fish vaccinated with the pCDNA3.1 vector alone, buffer alone, or unhandled fish (data shown in original references).

Five alternative delivery methods were tested as described in Corbeil *et al.* (2000a). These included: (a) intraperitoneal injection, (b) intrabuccal injection, (c) scarification of the skin by scratching the side of the fish from dorsal fin to abdomen with a 23-ga needle containing a droplet of concentrated vaccine solution (0.2 μg/ μl) on

the bevel, (d) cutaneous particle bombardment using a gene gun for a single delivery of DNA vaccine bound to 500 μg of 1 μm gold microcarriers, and (e) immersion for 40 min in water containing DNA-coated magnetic polystyrene beads. Each method was designed to deliver approximately 0.1 μg of DNA vaccine to each fish (Corbeil *et al.*, 2000a).

Challenge Experiments

After vaccination, fish were held for 4-6 wk and then challenged with virus in duplicate groups of 25-30 fish or triplicate groups of 15 fish. For standard challenges, fry (0.8-2.5 g fish) were immersed for 1 h in water containing 10^3-10^4 PFU of IHNV (WRAC or 220-90)/ml. Larger fish (17-160 g) were challenged by intraperitoneal injection with virus doses ranging from 10^4-10^7 PFU of IHNV strain 220-90/fish, as specified in the text. For the heterologous IHNV challenge experiment (Figure 2), each of the seven virus strains was used at the maximum immersion titers obtainable, which ranged from 10^3-10^7 PFU/ml due to variation in the titers generated during propagation of the different virus strains in cell culture (Corbeil *et al.*, 2000b). Each experiment also included groups of fish that were mock-challenged. Following exposure to the virus, duplicate or triplicate groups of 15-30 fish were held in 5-L tanks at 15°C and observed daily for mortality for 21-30 days.

Table 1. Relative percent survival (RPS)[a] of rainbow trout (*Oncorhynchus mykiss*) vaccinated with various doses of the pIHNVw-G DNA and subsequently exposed to a lethal IHNV challenge.

Host size	pIHNVw-G dose (μg)	RPS - Expt. 1 (%)	RPS - Expt. 2 (%)
Fry[b] (0.8-1.8 g)	1.0	100	nd
	0.1	97	90
	0.01	100	83
	0.001	nd	61
Subyearlings[c] (110-120 g)	10	100	100
	1.0	100	100
	0.1	nd	0

[a] RPS= [1-% mortality of pIHNVw-G vaccinated fry/% mortality of luciferase control vaccinated fry] ×100.

[b] Data for two fry experiments (expt.) is from Table 1 of Corbeil et al. (2000b). Duplicate groups of 30 fry were vaccinated by intramuscular injection and challenged 6 wk later by immersion in water containing 10^3-10^4 PFU of the WRAC strain of IHNV. Cumulative percent mortality (CPM) in the various pLuc control groups ranged from 24-60%; nd indicates "not done".

[c] Data for two subyearling trout experiments is from LaPatra et al. (2000). Groups of 20 subyearling trout were vaccinated by intramuscular injection and challenged 6 wk later by intraperitoneal injection with 10^5-10^6 PFU/fish of IHNV strain 220-90. CPM in pLuc control groups ranged from 40-90%.

Vaccine efficacy was determined by comparing the cumulative mortalities and survival times between groups of fish vaccinated with pIHNVw-G and those vaccinated with pLuc or other controls. Survival curves (% mortality over time) were estimated using the Kaplan-Meier method, and the logrank test was used to compare the survival curves (Statview, Abacus Concepts Inc., Berkeley, CA). Samples with P value <0.05 were considered to show a significant difference.

RESULTS

The DNA vaccine pIHNVw-G, containing the G gene of the IHNV WRAC strain, is shown in Figure 1, along with the control vaccine which differs only in having the luciferase marker gene in place of the G gene. In the experiments described below, various aspects of the efficacy of the pIHNVw-G vaccine were tested by comparing survival of pIHNVw-G vaccinated fish with survival of control pLuc vaccinated fish, when both were exposed to a lethal IHNV challenge *in vivo*.

Minimal Vaccine Dose Determination

In previous work, we demonstrated that the DNA vaccine pIHNVw-G provided strong, protective immunity in rainbow trout fry 4-6 wk after vaccination with doses of 1, 5, and 10 μg (Corbeil *et al.*, 1999). Experiments have subsequently been carried out to determine the minimal effective dose of pIHNVw-G in both fry (Corbeil *et al.*, 2000b) and in subyearling rainbow trout (LaPatra *et al.*, 2000). Results from these studies are summarized in Table 1, which shows data from two independent experiments in fish of each size. In rainbow trout fry (0.8-1.8 g), the pIHNVw-G vaccine at doses of 1.0 μg, 0.1 μg, or 0.01 μg reproducibly provided significant ($p<0.05$) protection [relative percent survival (RPS) 83-100%] against IHNV challenge. Fry vaccinated with a dose of 0.001 μg also showed significant protection relative to pLuc control vaccinated fish (RPS 61%), but the relative percent survival was lower than at higher vaccine doses, suggesting that this lowest dose is below the threshold for optimal efficacy of this vaccine. Although 0.01 was the minimal effective dose determined in this work, 0.1 μg was selected as the optimal dose for fry, to assure strong protection under the wide variety of conditions that could be encountered in aquaculture. This optimal dose was used for all subsequent experiments involving fry.

In subyearling rainbow trout (110-120g at vaccination), pIHNVw-G doses of 10 μg and 1 μg provided significant ($p<0.05$), complete protection (RPS 100%) against an injection virus challenge that caused 40-90% mortality in the pLuc control groups (LaPatra *et al.*, 2000). A smaller dose of 0.1 μg failed to provide any significant protection

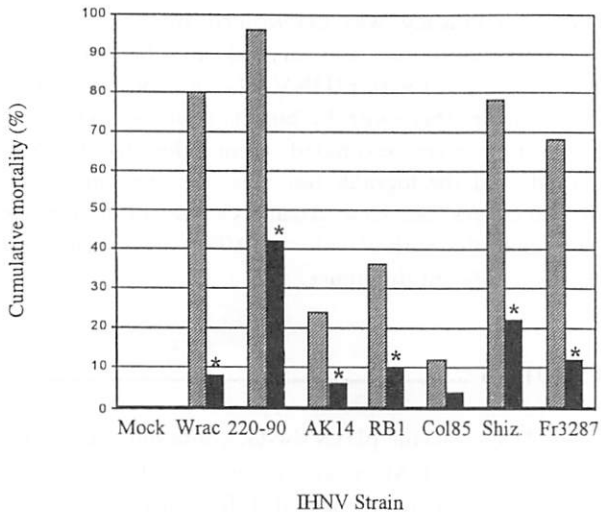

Figure 2. Ability of the pIHNVw-G DNA vaccine to protect rainbow trout fry against heterologous strains of IHN virus. The IHNV strain homologous to the pIHNVw-G vaccine (WRAC strain) and six heterologous IHNV strains (220-90, AK14, RB1, Col85, Shizuoka, and Fr3287; see text for information) were each used to challenge duplicate groups of 25 rainbow trout fry that had been vaccinated with 0.1 μg of the control vaccine pLuc (gray bars) or the DNA vaccine pIHNVw-G (black bars). Vaccinated fry (average weight 1.6 g) were held for 6 wk before immersion challenge. The data presented are average cumulative percent mortalities of duplicate groups, with pIHNVw-G groups shown adjacent to their respective pLuc control groups for each challenge virus strain. Asterisks indicate pIHNVw-G groups that were statistically different from their respective pLuc controls groups ($p<0.05$, logrank test).

relative to pLuc controls (RPS 0%). Thus, 1.0 μg is the minimal effective dose in fish of this size, and the threshold of efficacy was between 0.1 μg and 1.0 μg.

Protection Against Heterologous Challenge Strains of IHNV

The breadth of protection provided by the IHNV DNA vaccine was examined by challenging pIHNVw-G and pLuc vaccinated fry with seven different strains of IHNV (Corbeil *et al.*, 2000b). These strains included the WRAC strain, which is homologous to the vaccine, and six heterologous strains selected to represent the known genetic diversity of IHNV. The WRAC, RB1, and Col-85 strains, from Idaho, Oregon, and California, respectively, represent the three phylogenetic lineages of IHNV described by Nichol *et al.* (1995). The AK-14 strain was selected as a representative of IHNV in Alaska (Emmenegger *et al.*, 2000), and the 220-90 strain from Idaho was included because of its high virulence (LaPatra *et al.*, 1994). The Shizuoka strain isolated from rainbow trout in Japan in 1982 (Winton *et al.*, 1988) and strain 32-87 from rainbow trout in France in 1987 (Arkush *et al.*, 1989) were included as representatives of IHNV outside North America.

Six weeks after vaccination of rainbow trout fry (average weight 1.6 g) with 0.1 μg of pIHNVw-G or pLuc, duplicate groups of 25 fry were challenged with each of the seven IHNV strains. As shown in Figure 2, there was a wide range of mortality levels in the control groups, indicating phenotypic diversity in the virulence of these strains for rainbow trout. Fry vaccinated with pIHNVw-G showed significant protection ($p<0.05$) relative to pLuc control fish for six of the seven challenge strains. Only the Col-85 strain did not show statistically significant protection, due to the overall low mortality (12%) in the pLuc control groups for this strain. The highest mortality observed in pIHNVw-G fry occurred in groups challenged by immersion in 10^6 PFU/ml of the highly virulent 220-90 strain. This is a severe challenge that resulted in 96% cumulative percent mortality (CPM) in the pLuc control groups. In a later study using a lower challenge dose of 10^4 PFU/ml of strain 220-90, pLuc vaccinated fry showed a CPM of 80% and pIHNVw-G vaccinated fry had a CPM of 0% (Corbeil *et al.*, 2000a), confirming the ability of the pIHNVw-G vaccine to provide strong, significant ($p<0.05$) protection against this highly virulent heterologous strain of IHNV.

Efficacy of Various DNA Vaccine Delivery Methods

Although the pIHNVw-G DNA vaccine shows excellent efficacy when delivered by intramuscular (IM) injection, its ultimate utility in aquaculture will be limited until a mass delivery method is developed. As a first step toward this goal, five alternative methods of delivery were tested for their effects on vaccine efficacy (Corbeil *et al.*, 2000a). These included intraperitoneal injection (IP), intrabuccal injection (IB), scarification of the skin, immersion in water containing polystyrene beads coated with the DNA vaccine, and cutaneous particle bombardment using a gene gun. The protocols for each method were designed to deliver an optimal dose of 0.1 μg of DNA vaccine to each rainbow trout fry, and 4 wk later groups of fry were challenged by immersion in 10^4 PFU/ml of IHNV strain 220-90.

As shown in Figure 3, the only alternative method that provided protection comparable to the IM delivery route was cutaneous particle bombardment with the gene gun. IP injection also provided statistically significant protection ($p<0.05$), but the protection was not as strong as that observed with the IM and gene gun delivery routes. A histological time course study of target site tissue in fry vaccinated with the gene gun showed that the gold microcarrier particles were limited to the epidermal tissues, and that they were gradually removed from the integument over the course of at least 6 days as the outer

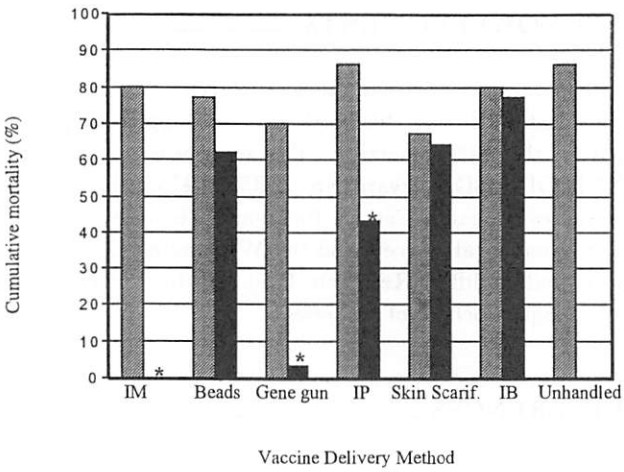

Figure 3. Efficacy of the pIHNVw-G DNA vaccine administered by various delivery methods. Six vaccination methods were used to deliver 0.1 μg of pLuc control (gray bars) or pIHNVw-G (black bars) to triplicate groups of 15 rainbow trout fry (average weight 1.8 g). The vaccination methods included: intramuscular injection (IM); immersion in water containing DNA-coated polystyrene beads (beads); cutaneous particle bombardment with a gene gun (Gene gun); intraperitoneal injection (IP); scarification of the skin (Skin Scarif); and intrabuccal administration (IB). Four weeks after vaccination, groups of fry were challenged by immersion in water containing 2.8×10^4 PFU of IHNV strain 220-90. The data presented are average cumulative percent mortalities of triplicate groups, with pIHNVw-G groups shown adjacent to their respective pLuc control groups for each vaccine delivery method. Asterisks indicate pIHNVw-G groups that were statistically different from their respective pLuc controls groups ($p<0.05$, logrank test).

cells were sloughed off (Corbeil *et al.*, 2000a).

Long-term Duration of the Protection Provided by the DNA Vaccine

In the first report describing pIHNVw-G (Corbeil *et al.*, 1999), groups of rainbow trout fry vaccinated with 1 μg of the vaccine showed significant protection against IHNV challenge at timepoints up to 80 days after vaccination. A 2-year study of the duration of protection provided by pIHNVw-G is currently in progress. This study was initiated by vaccinating groups of 500 rainbow trout fry (average weight 2.5g) with pIHNVw-G or pLuc at the optimal dose of 0.1 μg/fish. At timepoints 3, 6, and 13 months post-vaccination, duplicate groups of 25 fish from each vaccine treatment were tested for protection against an IP injection challenge with IHNV strain 220-90. Three months post-vaccination the pIHNVw-G vaccinated fish were completely protected (RPS 100%) relative to the pLuc control fish. At 6 and 13 months post-vaccination, there was still clearly significant protection ($p<0.05$, RPS 69% and 65%, respectively), but it was not as strong as that observed at 3 months (data not shown). Final results will not be available until the 24-month timepoint challenge has been carried out.

DISCUSSION

The combined results of these experiments document significant and reproducibly high efficacy for the pIHNVw-G DNA vaccine. The significant protection that is provided after administration of a single vaccine dose with no boosters is an important feature for its eventual use in aquaculture. The minimal effective dose that provided strong protection was 0.01 μg for fry and 1.0 μg for subyearling fish of 110-120g. These are much lower than the doses used in prior studies of IHNV DNA vaccines (Anderson *et al.*, 1996; Boudinot *et al.*, 1998; Corbeil *et al.*, 1999), indicating significantly better efficacy than was previously recognized. The same low minimal effective dose of 0.01 μg in rainbow trout fry was reported by Lorenzen *et al.* (2000) for a DNA vaccine against the important European rhabdovirus, viral hemorrhagic septicemia virus (VHSV). This suggests that high efficacy, in the form of low effective doses, may be a common feature of DNA vaccines against fish rhabdoviruses, or possibly of fish DNA vaccines in general. The low doses found to be effective for these DNA vaccines are advantageous both for minimizing the cost of future vaccines and for addressing regulatory concerns about exposing fish to excessive amounts of DNA. It is interesting to note that the minimum effective dose for fish weighing approximately 100g was 100-fold greater than the minimal dose for 1-g fry. This suggests a constant ratio of vaccine dose to host body weight. The ratio observed in our system is similar to ratios in reports of other DNA vaccines in mammalian hosts (Corbeil *et al.*, 2000b).

There have been many reports documenting phenotypic and genetic diversity among IHNV isolates from various sources (Winton *et al.* 1988; LaPatra *et al.*, 1994; Nichol *et al.*, 1995; Troyer *et al.*, 2000). However, serological cross-neutralization and cross-protection studies have suggested that all IHNV strains belong to a single major serotype, and that the G protein from one strain of IHNV can stimulate protective immunity in fish against heterologous IHNV strains (Engelking *et al.*, 1989; LaPatra *et al.*, 1994). Our demonstration that the IHNV DNA vaccine provides broad protection against a wide diversity of IHNV strains agrees with these previous observations and is another important feature of this vaccine.

At present, a major barrier to application of any DNA vaccine in fish is the need for an efficient mass immunization delivery method that will accommodate the large numbers of fish cultured in aquaculture settings. The most

valuable observation from our study of different delivery methods is that the gene gun was as effective as IM injection. This confirms the report of Gomez-Chiarri *et al.* (1996), who showed that both of these delivery methods worked well for DNA vaccine delivery to fish. Although the gene gun itself is not practical for mass immunization, the high efficacy it showed confirms that delivery of the vaccine to epidermal tissues can stimulate protective immunity. This provides a direction for future efforts, suggesting that mass delivery methods aimed at epidermal tissue might be successful. It is also useful that delivery of the DNA vaccine by IP injection provided partial protection. Since other vaccines in aquaculture are delivered by IP injection, it may be that the DNA vaccine could be delivered simultaneously with conventional vaccines by this route and provide dual protection to larger fish in some situations.

The study of the long term duration of protection has both practical and basic science value. At present, we know from the long term duration study that pIHNVw-G provides complete protection for up to 3 mon post-vaccination, and that there is still significant protection for over 1 year. In the next few months, we will know if this is still true 2 years after vaccination. From a practical standpoint, it is not necessary to protect rainbow trout for longer than about 6 mon, since they become relatively resistant to IHNV once they reach a size of approximately 20 g (LaPatra, unpublished observation). This is not true for some other host species such as Atlantic salmon, where an IHNV DNA vaccine could be useful to prevent disease losses in larger fish (Traxler *et al.*, 1999). From a basic science standpoint, the investigation of long term protection provides both insights and biological samples for examining the mechanism(s) of action of the DNA vaccine. At each timepoint in the long term duration study, in addition to challenge experiments to assess protection, additional fish have been sampled for serological, histological, and molecular analyses of the fate and effects of the DNA vaccine. Eventually this will enhance our understanding of this vaccine, and address regulatory and risk assessment concerns.

In summary, the pIHNVw-G DNA vaccine performed exceptionally well in each study described, illustrating the many positive features that are promising for its future use as a method for control of IHNV in aquaculture. The high efficacy demonstrated by this vaccine in fish surpasses the protection described for most DNA vaccines in mammalian hosts, making this an invaluable system for investigation of DNA vaccines in general. Finally, the DNA vaccine will be an important research tool for pioneering studies of the fish immune system. We hope that the knowledge gained with this vaccine will contribute toward development of control methods for many diseases that impact aquaculture, and toward improved aquatic animal health in general.

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CHARACTERIZATION OF COMPONENTS OF TYPE IV PILI AND TYPE II EXTRACELLULAR SECRETION IN *VIBRIO VULNIFICUS* AND DETERMINATION OF THEIR ROLE IN VIRULENCE

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ABSTRACT

Vibrio vulnificus expresses a number of cell-associated and extracellular products that may be involved in its ability to cause invasive disease in mammalian and marine vertebrate hosts. We have cloned and characterized four genes encoding products related to components of the type IV pilus biogenesis and general secretory (type II) pathways by complementation of a type IV prepilin peptidase (PilD) mutant of *Pseudomonas aeruginosa* with a *V. vulnificus* genomic library. One of the genes (*pilD*) encodes a protein homologous to PilD and other members of the type IV prepilin peptidase family, which completely restores this activity in a *P. aeruginosa* mutant deficient in the expression of PilD. The other genes (*pilA-C*) encode homologs of type IV pilus biogenesis components. These include the type IV pilin protein subunit (PilA) and two proteins essential for assembly of type IV pili (PilB and PilC). Phenotypic characterization of *V. vulnificus* with a mutation in *pilD* showed that PilD is required for the expression of surface pili. This mutant is also unable to secrete at least three extracellular degradative enzymes; the localization of one of these (cytolysin) to the periplasmic space indicates that these proteins are normally exported via the type II secretion pathway. Loss of PilD results in a significant decrease in Chinese hamster ovary cell cytotoxicity, adherence to HEp-2 cells, and virulence in a mouse model. Capsule formation and serum resistance are not affected in the PilD mutant, indicating that in addition to capsule, virulence of *V. vulnificus* requires type IV pili and/or extracellular secretion of degradative enzymes.

Key words: Vibrio, virulence, protein secretion, pili

INTRODUCTION

Vibrio vulnificus is a motile, Gram-negative, curved rod-shaped bacterium with a single polar flagellum. It is a naturally occuring, free-living inhabitant of estuarine and marine environments throughout the world, with a preference for tropical to subtropical climates. Currently, the species *V. vulnificus* is divided into two distinct biotypes based on phenotypic and host-range differences. Biotype 1 strains produce indole and ornithine decarboxylase, exhibit several immunologically distinct lipopolysaccharide (LPS) types, and are typically associated with shellfish colonization and human illness (for a recent review on the pathogenesis and epidemiology of *V. vulnificus* with a complete reference list, see Strom and Paranjpye, 2000) . Biotype 2 strains are negative for indole and ornithine decarboxylase production, express a common LPS type, and cause infections in a variety of marine vertebrates, particularly in cultured eels where it has caused significant economic losses in Japan, England, Spain, the Netherlands, and Denmark (Tison *et al.*, 1982; Krovacek *et al.*, 1994; Hoi *et al.*, 1998a, b). Although biotype 2 strains primarily cause infections in marine vertebrates, they are capable of causing opportunistic infections in mammalian hosts, including humans (Amaro and Biosca, 1996). In fact, there have been cases of transmission of *V. vulnificus*

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biotype 2 to humans resulting in severe infections (Veenstra *et al.*, 1993). Proven and suspected virulence determinants that may contribute to the ability of *V. vulnificus* to cause a severe and rapidly disseminating septicemia in susceptible hosts are the same for both biotypes. These include a polysaccharide capsule, several exoenzymes, and the ability to adhere to mucosal surfaces. Therefore, while much research in the pathogenesis of *V. vulnificus* has focused on biotype 1 strains, findings to date are significant for biotype 2 strains as well.

In this paper, we will review our work on characterization of genes encoding components of the type IV pilus biogenesis apparatus. In addition to their role in expression of pili, some of these genes are genetically, and in many cases functionally, linked to the machinery that controls extracellular secretion of many bacterial exoenzymes. This machinery has been labeled the terminal branch of the general secretory pathway or, more simply, the type II secretion system (Pugsley, 1993; Nunn, 1999).

TYPE IV PILI

Filamentous surface organelles called pili or fimbriae have been described on a variety of bacterial cells. Based on common features of these structures, they have been implicated in numerous cellular processes, including the recognition of receptors on eukaryotic cell membranes and interactions with other bacteria or a variety of inanimate surfaces. There are distinct families of pili, based on functional relatedness and several conserved features, including extensive sequence similarities of the subunit and accessory proteins (Strom and Lory, 1993). Type IV pili are a class of pili or fimbriae that are found only on Gram-negative bacteria with a preference for pathogens. These include the pili of *Pseudomonas aeruginosa, Aeromonas hydrophila, Neisseria gonorrhoeae, Moraxella bovis, and Vibrio cholerae*, to name a few. Shared common features include amino acid homology and a unique leader or signal sequence at the amino terminus in the precursor of the monomeric protein subunit of the pili, pilin (Figure 1A). This leader peptide is removed by endopeptidic cleavage, followed by *N*-methylation of the newly exposed amino-terminal amino acid, through the action of a bifunctional leader peptidase/*N*-methyltransferase ("type IV prepilin peptidase" or TFPP) before assembly of the pilin monomers into the pilus structure can take place (Nunn and Lory, 1991; Strom *et al.*, 1993). Besides their role as adhesins, some type IV pili have been shown to play additional roles in motility, DNA uptake, as phage receptors, and cell-signaling (Fussenegger *et al.*, 1997; Kim and Komano, 1997; Kallstrom *et al.*, 1998; Koomey, 1998; O'Toole and Kolter, 1998; Semmler *et al.*, 1999; Stone and Kwaik, 1999; Wall and Kaiser, 1999). Moreover, several type IV pilin-like proteins, which also require processing by a TFPP, have been identified and shown to play an as yet unidentified role in pilus assembly (Figure 1B).

TYPE II SECRETION

The third family of substrates processed by TFPPs are a group of proteins that are part of an apparatus in the Gram-negative cell envelope responsible for extracellular targeting of a class of proteins that are first secreted into the periplasm. This process has been named the type II secretion pathway (Pugsley, 1993; Nunn, 1999). The N-terminal regions of these proteins are highly homologous to the same region on the type IV pilins (Figure 1C). These proteins have been identified in several ways, including the linkage of their structural genes to those of other members of the secretion machinery, by demonstration of a pleiotrophic secretion defect in bacteria carrying mutated genes in their chromosome, or by low stringency hybridization with heterologous pilin gene probes. The genes encoding the four pilin-like proteins of the type II secretion pathway in *P. aeruginosa* were first identified by using mixed oligonucleotide probes spanning the consensus cleavage site recognized by the TFPP. Within the 14 genes that have now been identified to encode the type II secretion machinery, four are homologs of the type IV pilin subunit proteins, with the most homology in the N-terminal region where there is extensive similarity to the group A prepilins. There are a large number of bacterial species with such pilin-like groups of proteins, including *P. aeruginosa, Klebsiella oxytoca, Erwinia carotovora, E. chrysanthemi, A. hydrophila, A. salmonicida, V. cholerae*, and *Xanthomonas campestris* (for a complete discussion of TFPP substrates, see Strom and Lory, in press). A number of these also express type IV pili, including *P. aeruginosa, V. cholerae, V. vulnificus*, and *A. hydrophila*. In these organisms, a mutation in the TFPP gene results in a pleiotrophic mutation abolishing expression of type IV pili and extracellular secretion of proteins by the type II pathway (Strom *et al.*, 1991; Pepe *et al.*, 1996; Paranjpye *et al.*, 1998; Fullner and Mekalanos, 1999). All in all, the similarities between the biogenesis of type IV pili and type II protein secretion suggests that the latter may take place though cellular complexes resembling pili. Such organelles composed of the pilin homologs in the type II secretion pathway, termed "pseudopili," have been recently identified (Sauvonnet *et al.*, 2000).

GENETIC CHARACTERIZATION OF *V. VULNIFICUS*

The following describes a study performed in our laboratory on the isolation of components of type IV pilus biogenesis and type II protein secretion in *V. vulnificus*. The work characterizing the *V. vulnificus* type IV leader

A. Type IV pilins

```
Group A                                                    ▼
  P. aeruginosa PilA                    MetLys   AlaGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
  P. syringae PilA                      Met   AsnAlaGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
  M. bovis TfpQ                         Met   AsnAlaGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
  M. nonliquefaciens TfpA               Met   AsnAlaGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
  D. nodosus FimA                       MetLysSerLeuGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
  N. gonorrhoeae PilE                   MetAsnThrLeuGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
  N. meningitidis PilE                  MetAsnThrLeuGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
  E. corrodens EcpA                     MetLysGlnValGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
  X. campestris PilA                    MetLys   LysGlnAsnGly PheThrLeuIleGluLeuMetIleValIle-
  A. hydrophila TapA                    MetLys   LysGlnSerGly PheThrLeuIleGluLeuMetIleValVal-
  A. salmonicida TapA                   MetLys   LysGlnSerGly PheThrLeuIleGluLeuMetIleValVal-
  A. veronii bv sobria TapA             MetLys   LysGlnSerGly PheThrLeuIleGluLeuMetIleValVal-
  P. putida PilA                        MetLys   GlyGlnArgGly IleThrLeuIleGluLeuMetIleValVal-
  V. cholerae PilA   MetLys   AlaTyrLysAsnLysGlnGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
  V. vulnficus VvpA  MetLysLysLeuAspLysThrLysLysGlnGlnGly PheThrLeuIleGluLeuMetIleValVal-

Group B
  V. cholerae TcpA      MetGln..16..LysLysThrGlyGlnGluGly MetThrLeuLeuGluValIleIleValLeu-
  E. coli BfpA          MetVal.. 9..AsnLysLysTyrGluLysGly LeuSerLeuIleGluSerAlaMetValLeu-
```

B. Pilin-like proteins required for type IV pilus biogenesis

```
  P. aeruginosa PilE                    MetArgThrArgGlyLysGly PheThrLeuLeuGluMetValValValVal-
  P. aeruginosa PilV    MetLeu..5...ArgSerLeuHisGlnSerGly PheSerMetIleGluValLeuValAlaLeu-
  P. aeruginosa FimU          MetSerTyrArgSerAsnSerThrGly PheThrLeuIleGluLeuLeuIleIleVal-
  P. aeruginosa FimT             MetValGluArgSerGlnArgAla LeuThrLeuThrGluLeuLeuPheAlaLeu-
  N. meningitidis FimT           MetCysThrArgLysGlnGlnGly PheThrLeuThrGluLeuLeuIleValMet-
  N. meningitidis PilV  MetAsn..7...ArgLeuLysSerGlnSerGly MetAlaLeuIleGluValLeuValAlaMet-
```

C. Pilin-like proteins required for extracellular (type 2) protein secretion

```
  P. aeruginosa                                            ▼
   XcpT                           LeuGlnArgArgGlnGlnSerGly PheThrLeuIleGluIleMetValValVal-
   XcpU                                MetArgAlaSerArgGly PheThrLeuIleGluLeuMetValValMet-
   XcpV                                MetLysArgAlaArgGly PheThrLeuLeuGluValLeuValAlaLeu-
   XcpW                                MetArgLeuGlnArgGly PheThrLeuLeuGluLeuLeuIleAlaIle-

  K. oxytoca
   PulG                                   MetGlnArgGlnArgGly PheThrLeuLeuGluIleMetValValIle-
   PulH                                      ValArgGlnArgGly PheThrLeuLeuGluMetMetLeuIleLeu-
   PulI                                   MetLysLysGlnSerGly MetThrLeuIleGluValMetValAlaLeu-
   PulJ                                MetIleArgArgSerSerGly PheThrLeuValGluMetLeuLeuAlaLeu-

  E. chrysanthemi
   OutG                                   MetGluArgArgGlnArgGly PheThrLeuLeuGluIleMetValValIle-
   OutH                                        ValArgGlnArgGly PheThrLeuLeuGluIleMetLeuValVal-
   OutI                                     MetLysGlnGlnGly MetThrLeuLeuGluValMetValAlaLeu-
   OutJ                                ValLysGlnProGluArgGly PheThrLeuLeuGluValMetLeuAlaLeu-

  E. carotovora
   OutG            MetGlnGlnSerGln  6  SerTyrGlyGlnSerGly PheThrLeuLeuGluIleMetValValIle-
   OutH                    MetLysArgSerThrArgLysGlnGlnGly PheThrLeuLeuGluMetMetLeuValVal-
   OutI                                MetArgArgGlnLysGly MetThrLeuValGluValLeuValAlaLeu-
   OutJ            MetSerSerLysThr 14  GlnGluArgGlnGlnGly PheThrLeuLeuGluMetIleLeuAlaIle-

  X. campestris
   XpsG            MetIleLysArgSer..6..ArgAlaGlyGlnAlaGly MetSerLeuLeuGluIleIleIleValIle-
   XpsH            MetArgValAlaArg.13..ArgArgGlnLeuArgGly SerSerLeuLeuGluMetLeuLeuValIle-
   XpsI                                   MetLysHisGlnArgGly TyrSerLeuIleGluValIleValAlaPhe-
   XpsJ                                MetArgProArgAlaAlaGly PheThrLeuIleGluValLeuLeuAlaThr-

  A. hydrophila
   ExeG                                MetGlnLysArgArgGlnSerGly PheThrLeuLeuGluValMetValValIle-
   ExeH                                MetArgArgHisArgGlnSerGly PheThrLeuLeuGluValLeuLeuValAla-
   ExeI                                      MetAspAlaArgGly MetThrLeuLeuGluValMetValAlaLeu-
   ExeJ                                MetLysSerArgGlnGlyGly PheThrLeuLeuGluMetLeuValAlaIle-
```

Figure 1. Amino acid sequences of the amino-terminal domains. including the leader peptides, of some type IV pilins and type IV pilin-like proteins involved in pilus biogenesis. The q denotes the cleavage site where TFPPs proteolytically remove the leader peptides. **A.** The group A and group B type IV prepilins, with Genbank protein accession number in parentheses: *P. aeruginosa* PilA (P02973), *P. syringae* PilA (AAA25974), *Moraxella bovis* TcpQ (A55851), *M. nonliquefaciens* TfpA (AAA25310), *Dichelobacter nodosus* FimA (P04953), *Neisseria gonorrhoeae* PilE (AAC38436), *N. meningitidis* PilE (S55496), *Eikenella corrodens* EcpA (CAA78250), *Xanthomonas campestris* PilA (S52692), *Aeromonas hydrophila* TapA (P45791), *A. salmonicida* TapA (AAC23566), *A. veronii* biovar *sobria* TapA (AAD09352), *Pseudomonas putida* PilA (D36961), *Vibrio cholerae* PilA (AAD21029), *V. vulnificus* PilA (M. S Strom and R. N. Paranjpye, unpublished data) , *V. cholerae* TcpA (P23024), *Escherichia coli* BfpA (P33553). **B.** Pilin-like proteins involved in type IV pilus biogenesis, with Genbank protein accession number in parentheses: *P. aeruginosa* PilE (S54700), *P. aeruginosa* PilV (S77594), *P. aeruginosa* FimU (AAB39271), *P. aeruginosa* FimT (AAB39270), *N. meningitidis* FimT (AAF62321), *N. meningitidis* PilV (AAF41297). **C.** Pilin-like proteins involved in type II protein secretion and that require processing by a TFPP, with Genbank protein accession number in parentheses: *P. aeruginosa* XcpTUVW (M80792 and X62666), *K. oxytoca* PulGHIJ (M32613), *E. chrysanthemi* OutGHIJ (L02214), *E. carotovora* OutGHIJ (X70049), *X. campestris* XpsGHIJ (L02630), and *A. hydrophila* ExeGHIJ (X66504).

peptidase PilD (formerly VvpD) was recently published (Paranjpye *et al.*, 1998) and what follows is largely a review of that work along with a discussion of future directions in our continuing study of the role of type IV pili in this pathogen.

Isolation of genes encoding components of type IV pilus biogenesis.

As discussed earlier, type IV pili are responsible for a form of motility called "twitching motility" in several Gram-negative bacteria, and is most studied in *Pseudomonas aeruginosa*. Twitching motility is an irregular movement on semi-solid surfaces and is a result of assembly of the pilin protein into the pilus structure, followed by a depolymerization of the structures (Merz *et al.*, 2000). Colonies of *P. aeruginosa* have a rough appearance with an irregular edge due to this type of motility. *P. aeruginosa* mutants unable to express normal type IV pili do not have this type of movement and colonies are smooth and circular with a sharp edge. One such class of mutants are those unable to express the type IV prepilin peptidase (TFPP), as this product is required for proteolytic cleavage and removal of the precursor pilin (or prepilin) leader sequence prior to assembly of the pilin monomers into pili. A secondary screen for the non-piliated phenotype that was used for confirmation utilizes sensitivity to a *P. aeruginosa* bacteriophage, PO4. This lytic phage binds to the type IV pilus of *P. aeruginosa*. Non-piliated mutant strains are resistant to the phage, while normal piliated strains are lysed. The use of these screening methods are completely described elsewhere (Nunn and Lory, 1991; Strom and Lory, 1991; Strom *et al.*, 1991; Pepe *et al.*, 1996). A third phenotype used was observation of hemolysis of sheep erythrocytes on blood agar plates. This hemolysis is the result of *P. aeruginosa* phospholipase C, secreted via the type II secretion pathway as discussed previously (Strom *et al.*, 1991). PilD mutants of *P. aeruginosa* do not have zones of hemolysis around colonies on blood agar. These phenotypes were exploited to clone the gene encoding the TFPP from *V. vulnificus* in the following manner.

A cosmid library of *V. vulnificus* DNA on a compatible vector was introduced by conjugation into a *P. aeruginosa* strain carrying a mutation in *pilD*, and the resulting colonies screened for restoration of twitching motility (Figure 2) and by zones of hemolysis on sheep blood agar. Using both phenotypes to observe colonies, complementing clones were isolated. Reversion to phage sensitivity was also confirmed (Figure 2). Sequencing demonstrated the presence of a four-gene cluster that encodes homologues of *pilA*, *pilB*, *pilC*, and *pilD*. These genes are highly homologous to their *P. aeruginosa* counterparts, and were originally designated *vvpABCD* where "vvp" stood for *V. vulnificus* pili. These genes hence will be referred to as *pilABCD* to avoid confusion with the genetic nomenclature for genes encoding factors involved in expression of the *V. vulnificus* metalloprotease. The only difference in organization between the two sets of genes is the direction of transcription of the *pilA* gene. In *P. aeruginosa*, *pilA* is oriented in the opposite direction of *pilBCD*, while in *V. vulnificus*, all four

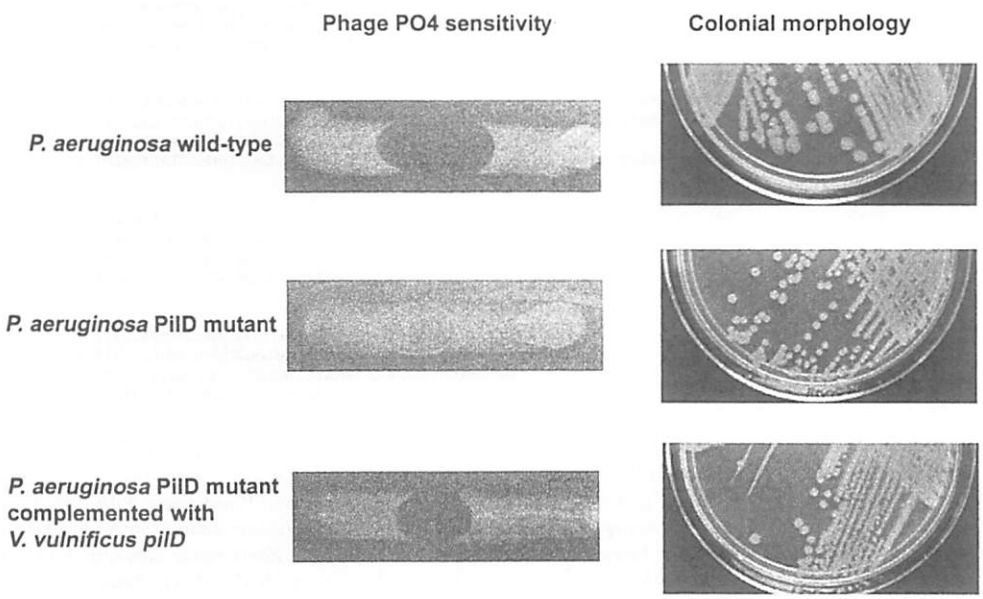

Figure 2. Complementation of the *P. aeruginosa* PilD mutant used to isolate the *V. vulnificus pilD* gene by complementation. Wild-type *P. aeruginosa* is sensitive to the pilus-binding phage PO_4, while a strain without functional PilD is not (first column). Similarly, wild-type *P. aeruginosa* has twitching motility resulting in a distinct colonial morphology (flatter, rough-looking colonies). In contrast, non-piliated *P. aeruginosa* forms smooth and rounder colonies (second column).

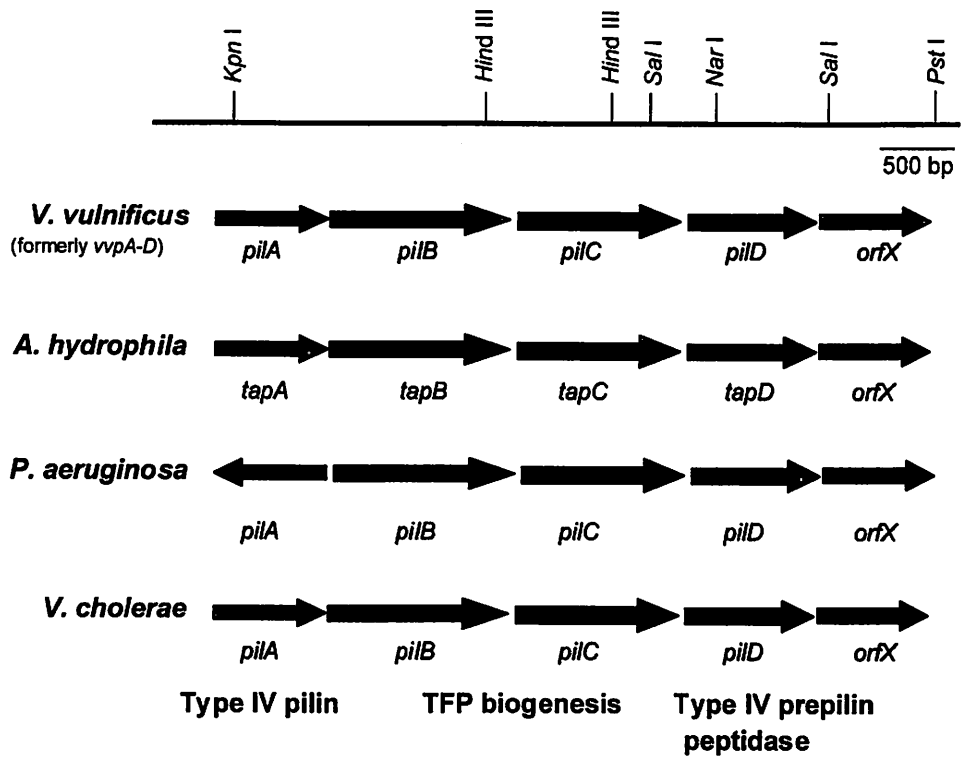

Figure 3. Genetic organization and partial restriction map of the sequenced *pilABCD* genes from *V. vulnificus* and comparison to other Gram-negative bacteria that have similar organization of these four genes. In addition, all have a fifth gene encoding an open reading frame of unknown function (*orfX*) immediately downstream of the gene encoding the TFPP.

genes are transcribed in the same direction (Figure 3). This finding is similar to what we found for the homologous genes in *Aeromonas hydrophila* (Pepe *et al.*, 1996) and *A. salmonicida* (C. M. Pepe and M. S. Strom, unpublished results), and what has been demonstrated elsewhere for *V. cholerae* (Fullner and Mekalanos, 1999). *V. vulnificus* PilD shows 59% similarity and 52% identity to PilD of *P. aeruginosa*, while *V. vulnificus* PilA shows 47% similarity and 42% identity to *P. aeruginosa* PilA (analysis using GCG Pileup, Wisconsin Genetic Computer Group). The cloned *V. vulnificus pilD* was used to construct an isogenic mutant strain by interrupting the coding sequence of the gene via insertion of an antibiotic resistance cassette, followed by conjugation into wild-type *V. vulnificus* and selection for the double recombinant (allelic exchange). In the experiments described below, the wild-type *V. vulnificus* strain was compared to this mutant and a complemented mutant strain that carried *pilD* on a plasmid where PilD is expressed *in trans* from an inducible promoter.

Characterization of the *V. vulnificus* TFPP.

In order to verify that the *V. vulnificus* PilD is truly a TFPP, an experiment was performed to show that *V. vulnificus PilD* can cleave *P. aeruginosa* prepilin *in vitro*. Taking advantage of the fact that TFPPs are integral membrane proteins (Nunn and Lory, 1991; Reeves *et al.*, 1994), an *in vitro* cleavage assay (Strom *et al.*, 1994) was carried out by mixing *P. aeruginosa* prepilin (isolated from a *P. aeruginosa pilD* mutant strain) with membrane material extracted from *V. vulnificus* wild-type, mutant, and complemented mutant strains. As shown in Figure 4, membranes from wild-type *V. vulnificus* contain an activity that results in the removal of the leader peptide from the prepilin (Figure 4, lane 2), while the mutant strain does not (Figure 4, lane 3). This is demonstrated by the faster electrophoretic mobility of the pilin protein on sodium dodecyl sulfate-polyacrylamide gels. This activity is restored in the complemented mutant strain (Figure 4, lane 4). Obviously, these results do not unequivocally

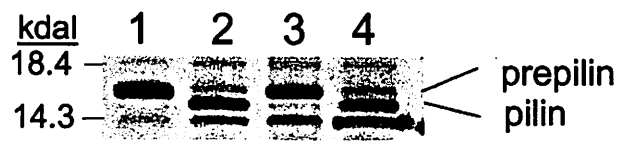

Figure 4. Sodium dodecyl sulfate-15% polyacrylamide gel showing *in vitro* activity of *V. vulnificus* PilD (contained in membrane preparations) on *P. aeruginosa* prepilin substrate. Lanes: 1, prepilin substrate only; 2, wild-type *V. vulnificus*; 3, *V. vulnificus pilD* mutant; 4, complemented *V. vulnificus pilD* mutant.

demonstrate that *V. vulnificus* PilD is removing the leader peptide at the precise site cleaved by *P. aeruginosa* PilD (MKAQKG ▼ FTLIE where ▼ is the cleavage site). However, there has been sufficient accumulation of evidence, obtained through N-terminal sequencing of processed (cleaved) pilins, to know that cleavage between the glycine and phenylalanine residues is required before assembly of the pilin monomers into the pilus structure can take place (Nunn and Lory, 1993; Strom *et al.*, 1993). Since the *P. aeruginosa pilD* strain is complemented by *V. vulnificus* PilD, one can presume that *V. vulnificus* PilD is also a typical type IV prepilin peptidase.

A series of experiments were then performed to show that *V. vulnificus* secretes several exoenzymes through the type II pathway. This analysis took advantage of the fact that a periplasmic space localization of proteins normally exported extracellularly, in a bacterium with a mutation in a TFPP gene, defines the type II pathway (Strom *et al.*, 1991; Pugsley, 1993; Pepe *et al.*, 1996). The localization of three *V. vulnificus* enzymes, the cytolysin/hemolysin, protease, and chitinase, were examined. For examination of the localization of the cytolysin/hemolysin, cultures of cells from *V. vulnificus* wild-type, *pilD* mutant and complemented mutant were fractionated into supernatant (extracellular), periplasmic, and cytoplasmic fractions. These extracts were then used to lyse sheep erythrocytes in a modified plate hemolysin assay. As shown in Table 1, the hemolytic activity from the wild-type and complemented mutant strains was located largely in the extracellular fraction. However, this activity was localized in the periplasmic space in the *pilD* mutant. A control strain carrying a mutation in the gene encoding the cytolysin, *vvhA*, was included to demonstrate that this hemolytic activity was the result of the cytolysin protein. Similarly, activities consistent with a protease and chitinase were also detected in supernatants from wild-type and complemented strain cultures, but not in supernatants from cultures of the *pilD* mutant. Taken together, these results indicate that a functional type II secretion system exists in *V. vulnificus* and that secretion of the cytolysin/hemolysin, protease, and chitinase follow this pathway.

To determine whether pili observed on the surface of *V. vulnificus* are of the type IV class, the wild-type, *pilD* mutant, and complemented mutant strains were examined by transmission electron microsopy. Long, filamentous structures with typical pilus morphology were easily observed on the wild-type and complemented strains, while no pili were seen on the surface of the mutant strain (Figure 5). This strongly suggests that these pili are type IV since in the absence of a TFPP, they are not expressed.

It is known that the specific virulence functions ascribed to the capsule of *V. vulnificus* include inhibition of phagocytosis by macrophages and resistance to the bactericidal effects of serum. Since the *V. vulnificus pilD* strain demonstrates a major secretion defect, there was some concern that the mutant strain was defective in capsular expression or localization. However, the presence of an intact capsule was confirmed in several ways. First, there was no visible difference in the opacity of colonies on various agar-based media. In general, non-encapsulated strains form translucent colonies that are easy to distinguish from opaque, encapsulated strains. Likewise, hydrophobicity in ammonium sulfate, another assay that easily distinguishes encapsulated and non-encapsulated strains, was not measurably different between the wild-type and mutant strains. Direct staining of the capsule with ruthenium red and examination of ultra-thin sections by transmission electron microscopy revealed no difference in the thin layer of exopolysaccharide on the cell surface (Figure 5). And last, assays to determine the resistance or sensitivity to the bactericidal effects of serum clearly showed that the *pilD* mutant strain was as resistant to treatment with human serum as the wild-type (Table 1).

It has been observed that culture supernatants from *V. vulnificus* are cytotoxic to a variety of cultured epithelial cells. This activity has long been attributed to the activity of the 51 kdal cytolysin, which is responsible for hemolysis of erythrocytes and shows cytolytic activity *in vitro* (Gray and Kreger, 1985, 1986). This assay involved adding filtered culture supernatants from overnight cultures to monolayers of Chinese hamster ovary (CHO) cells. Since we previously demonstrated that there was no secretion of hemolytic activity in the *pilD* mutant strain, we assumed that culture supernatants of the mutant would be less cytotoxic to the CHO cells. This indeed was the case, as supernatants from the mutant were demonstrably less cytotoxic than wild-type *V. vulnificus* (Table 1). Full cytotoxicity was restored by complementation of the mutant with *pilD*. As with the hemolytic assay, a control strain with a mutation in the gene encoding the cytolysin (*vvhA*) was included in the assay. Surprisingly, this strain was just as cytotoxic in the CHO cell assay as wild-type, suggesting that cytotoxicity of culture supernatants is due to a combination of other, as yet unidentified, degradative and cytotoxic enzyme (s). Contrary to what had been believed, tissue culture cytotoxicity does not necessarily include the *vvhA*-encoded cytolysin.

Next we wanted to determine whether pili or other type II secretion product plays a role in adherence to epithelial cells. Specific adherence is a prelude to colonization of host tissues and is considered a virulence factor. To determine specific adherence of *V. vulnificus* to epithelial cells, we developed a quantitative and reproducible adherence assay using monolayers of HEp-2 cells, a human epithelial cell line. In the assay, washed *V. vulnificus* cells are added at numbers no greater than 10 colony-forming units per epithelial cell in order to avoid the rapid cytotoxic effect that otherwise quickly destroys the integrity of tissue culture monolayers. After a short incubation, the monolayers are washed to remove non-adherent bacteria.

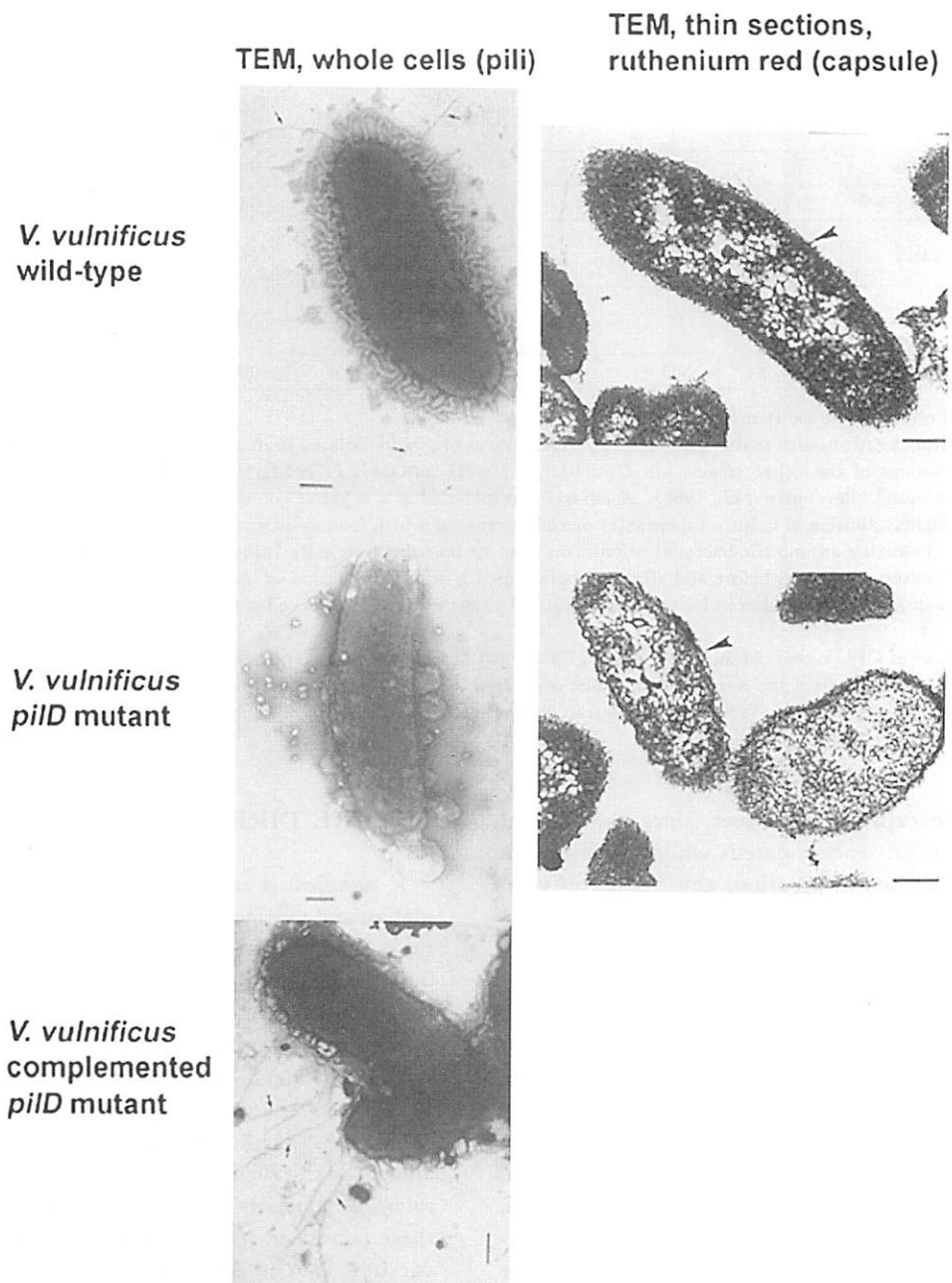

Figure 5. Transmission electron micrographs of whole cells and ruthenium red-stained thin sections of wild-type, *pilD* mutant, and complemented *pilD* mutant strains of *V. vulnificus*. In the first column, visible pili are denoted by the arrows for the wild-type and complemented mutant strains. In the second column, the arrowheads mark the thin exopolysaccharide capsule that is clearly visible at the periphery of both wild-type and mutant strain cells (bar=200 nm).

V. vulnificus is extremely susceptible to lysis with a variety of non-ionic detergents. Therefore, instead of a standard detergent lysis of the monolayers to release adherent bacteria, *V. vulnificus* cells are resuspended by mechanical agitation of the monolayer. As shown in Table 1, approximately 9% of wild-type *V. vulnificus* cells adhere to HEp-2 cells, while the *pilD* mutant strain is significantly less adherent with less than 0.5% of input bacteria remaining associated with the monolayers. Complementation of the mutant with a functional *pilD* restored adherence to values close to wild-type levels. These results suggest that either the type IV pili or a type II secretion product (or products or both) are required for adherence to the HEp-2 cell line. A more definitive determination of the role of type IV pili in the adherence awaits analysis of specific mutants in the gene encoding the type IV pilin subunit protein.

As discussed earlier, the only proven virulence factor identified for *V. vulnificus* prior to this work was the

Table 1. Summary of phenotpyes observed in the *Vibrio vulnificus* pilD mutant.

	Hemolytic index[2]			Extracellular secretion[3]		Serum resistance[4]				
Strain[1]	Supernatant	Periplasm	Cytoplasm	Protease	Chitinase	Normal	Heat-inactivated	CHO cell cytoxicity[5]	HEp-2 cell adherence (%)[6]	Mouse LD_{50}[7]
Wild-type	2048	0	0	+	+	0.46	0.44	64	9.2±2.1	9.4×10^3
pilD mutant	8	272	4	-	-	0.93	0.65	<2	0.3±0.1	1.1×10^6
Complemented *pilD* mutant	2048	16	4	+	+	ND	ND	64	4.5±2.0	2.1×10^5
Cytolysin (*vvhA*) mutant	0	0	0	ND	ND	ND	ND	64	9.0±3.6	ND
E. coli S17	ND	ND	ND	ND	ND	>6.4	0.71	ND	ND	ND

For complete details of all assays, see Paranjpye *et al.* (1998).

[1] The wild-type *V. vulnificus* strain used in this study is C7184/OP. It was originally isolated from a human patient and obtained from J. D. Oliver. The complete designations of the other strains are C7184-$D_{12}\Omega$ (pilD mutant), C7184-$D_{12}\Omega$/pRPD1 (complemented *pilD* mutant, and C7184-402K (*vvhA* mutant) (Paranjpye *et al.*, 1998). *E. coli* S17 was included as a negative control only for the serum resistance assay.

[2] Reciprocal of the highest dilution of culture supernatant or cell fraction at which hemolysis was observed (average of two assays).

[3] Detected by zones of clearing around the bacterial colonies on agar media containing milk (protease) or colloidal chitin (chitinase).

[4] Log_{10} reduction of bacterial numbers before and after incubation for 1 h at 37℃ in normal or heat-inactivated serum.

[5] Reciprocal of the highest dilution of filtered bacterial supernatants to cause typical CHO cell cytotoxicity (cell lysis, rounding, and detachment of monolayer) after 6 h.

[6] Presented as (number of CFU recovered/number of input CFU) $\times 100 \pm$ standard deviation. Values for wild-type or complemented mutant strain are significantly different from the mutant strain when compared by Tukey-Kramer analysis at $P \leq 0.5$.

[7] Calculated LD_{50} (Reed and Muench, 1938) in iron-dextran treated mice.

ND, not done

exopolysaccharide capsule. However, since the mutant *pilD* strain remained encapsulated while deficient in secretion of proteins and in piliation, any change in virulence will aid the identification and subsequent characterization of additional virulence determinants. Using the iron dextran-treated mouse model, the LD_{50} of the *pilD V. vulnificus* mutant strain was greater than 2-logs higher than the wild-type strain (Table 1). This difference was not due to any defect in *in vivo* growth of the mutant strain since equivalent numbers of bacteria were recovered from the spleens of infected mice 24 h postinfection. Complementation of the mutant strain resulted in partial restoration of virulence towards wild-type levels. Therefore, as with the results for specific cellular adherence, a combination of type IV pili or secreted proteins are most probably involved in the pathogenesis of *V. vulnificus*. Clarification will require the construction of isogenic mutant strains with defects in individual genes encoding some of these factors. However, it should be pointed out here that previous studies on a mutant unable to express the cytolysin (VvhA) showed it was no less virulent than its wild-type counterpart (Wright and Morris, 1991). While this is analogous to our results that demonstrated that a *vvhA* mutant is just as cytotoxic as its wild-type counterpart, it also demonstrates the possibility that many single gene mutants of *V. vulnificus* may not have decreased virulence. As a rule, pathogenesis is the result of the synergistic effect of many factors.

FUTURE DIRECTIONS

V. vulnificus is an opportunistic pathogen in both mammalian and marine vertebrate hosts. While this broad host range is somewhat predicated by strain differences involving biotype and lipopolysaccharide composition, the list of other possible and proven virulence determinants expressed by *V. vulnificus* is similar between biotype 1 and 2 strains. Indeed like most pathogens, virulence of *V. vulnificus* is undoubtedly multifactorial and requires a complex interaction of many factors to colonize, multiply, and cause the tissue destruction that is the hallmark of *V. vulnificus* infections. Therefore, a better characterization of these factors is required before improved treatments or vaccines can be developed. Such an understanding is important for both biotypes of this pathogen, and is required whether the discussion involves infections in human or marine vertebrate hosts. It should also be pointed out that there is a more direct and practical application for the information developed on the adherence or colonization mechanisms used by *V. vulnificus*. It is possible that the true role of such "virulence" factors may be to allow the organism to specifically adhere and persist in molluscan shellfish tissue. Therefore, the identification and understanding of these mechanisms may lead to the development of better ways to depurate shellfish of the organism prior to marketing, through the use of competitive inhibitors or other techniques.

We are currently characterizing the expression and localization of a type IV pilin, the gene for which (*pilA*) is

located upstream (Figure 3) of the gene encoding the TFPP PilD. In preliminary surveys, we have shown that the *pilA* and *pilD* genes are found in all biotype 1 strains surveyed (unpublished results), and we hope to extend this to include biotype 2 strains as well. Other planned research includes a determination of the role of this protein in expression of a type IV pilus, its role in adherence to mammalian cells, and virulence in the iron-dextran treated mouse model. Future work includes a determination of the roles of these factors in molluscan shellfish adherence, both in experiments utilizing primary cells harvested from oysters, as well as in the whole animal.

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COLD-WATER DISEASE OF COHO SALMON IN JAPAN

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ABSTRACT

A large number of coho salmon (*Oncorhynchus kisutch*) eggs have been imported annually from the Pacific coast of North America. Outbreaks of cold-water disease in coho salmon in Japan occurred only in the fry originating from the imported eggs which showed positive for *Flavobacterium psychrophilum*, despite the fact that the eggs were disinfected with 50 ppm povidone-iodine for 15 min. Experimental infection of eggs with *F. psychrophilum* was successful only by immersing the fertilized eggs in a suspension of the bacteria at a concentration of 1.0×10^8 CFU/mL before water-hardening. *F. psychrophilum* was isolated from the egg contents and the viable bacterial counts ranged from 10^3 to 10^7 CFU/g. Observation on the frozen sections of the infected eggs at eyed stage stained by IFAT revealed that many *F. psychrophilum* cells were located within the eggs. It was concluded that *F. psychrophilum* entered the eggs during the water-hardening stage. The povidone-iodine treatment was ineffective to eliminate *F. psychrophilum* from the experimentally infected eggs. Introducing the domestic eggs instead of the imported eggs into the hatcheries was effective for controlling the disease.

Key words: waterborne transmission, *Flavobacterium psychrophilum*, cold-water disease coho salmon, egg, *Oncorhynchus kisutch*, Japan.

INTRODUCTION

Flavobacterium psychrophilum, the causative agent of cold-water disease, was originally isolated from coho salmon in the USA in 1948 (Borg, 1960). The bacterium has also been isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in Europe (Bernardet and Kerouault, 1989). In Japan, outbreaks of cold-water disease have often occurred in coho salmon (*O. kisutch*) hatcheries since the middle of the 1980s (Wakabayashi *et al.*, 1991). The disease has also been reported in rainbow trout, ayu (*Plecoglossus altivelis*) (Wakabayashi *et al.*, 1994) and pale chub (*Zacco platypus*) (Iida and Mizokami, 1996). The bacterium has also been detected from wild masu slmon (*O. masou*) and other wild fish (Amita *et al.*, 2000).

IMPORTED EGGS RESPONSIBLE FOR THE OUTBREAKS OF COLD-WATER DISEASE

The intensive marine farming of coho salmon in Japan began in November 1974 (Mahnken, 1991). Subsequently, a large number of coho salmon eggs have been imported annually from the Pacific coast of North America, due to lack of the natural stock of this species. Therefore, the imported eggs have been suspected to be an infection source of outbreaks of cold-water disease in coho salmon hatcheries in Japan. Izumi and Wakabayashi (1997) reported that five of nine lots of coho salmon eggs imported from the USA in December 1995 were found to be positive for *F. psychrophilum* by polymerase chain reaction and four of these five lots were also positive by cultivation on TYE agar (tryptone 0.4%, yeast extract 0.05%, $CaCl_2 \cdot 2H_2O$ 0.02%, $MgSO_4 \cdot 7H_2O$ 0.05%, agar 1.5%, pH 7.2). Kumagai and Takahashi (1997) reported that all of the imported eggs were disinfected with 50 ppm iodine for 15 min at the hatcheries just after arriving there, but the outbreaks of cold-water disease occurred only among fry originating from some lots of imported eggs positive for *F. psychrophilum*. In contrast, there was neither detection of the pathogen nor occurrence of cold-water disease among the lots of domestic eggs and fry. Therefore, it is supposed that the disease outbreaks were caused by the pathogen being accompanied by the imported eggs, and that some *F.*

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psychrophilum is still alive after disinfection with povidon-iodine.

EXPERIMENTAL INFECTION OF COHO SALMON EGGS WITH *F. PSYCHROPHILUM*

Kumagai *et al.* (2000) determined that salmonid eggs were infected with *F. psychrophilum* by immersion challenge. Fertilized coho salmon eggs were immersed in diluted broth culture of *F. psychrophilum* (1.0×10^8, 1.0×10^6, 1.0×10^4 CFU/mL) for 30 min before water-hardening, after water-hardening and at eyed-egg stages. Just after exposure to the bacteria, the eggs were disinfected with povidone-iodine, and then discretely incubated in flowing water. After 7-28 days of incubation, *F. psychrophilum* was isolated only from the egg group which had been exposed to 1.0×10^8 CFU/mL before water-hardening (Table 1). Experimental infection of eggs with *F. psychrophilum* was successful only by immersing the fertilized eggs in a suspension of the bacteria at a concentration of 1.0×10^8 CFU/mL before water-hardening (Kumagai *et al.*, 2000).

Yousif *et al.* (1994) reported that *A. salmonicida* was susceptible to lysozyme at a concentration to that found in the yolk of salmonid eggs, but *Renibacterium salmoninarum* was not susceptible. They considered that resistance of *R. salmoninarum* to lysozyme might explain why this organism survives in salmonid eggs well enough to be vertically transmitted. Brown *et al.* (1997) indicated that *F. psychrophilum* was not as susceptible to lysozyme as *A. salmonicida*. Kumagai *et al.* (2000) reported that *F. psychrophilum* infected the eggs by experimental infection, while *A. salmonicida* did not, and that this result may be related to the difference of susceptibility to lysozyme between the two bacteria.

INEFFECTIVENESS OF IODOPHORE TREATMENT IN DISINFECTING SALMONID EGGS

Kumagai *et al.* (1998) attempted to determine whether an iodophore treatment could effectively disinfect the experimentally infected eggs. The disinfection of the experimentally infected eggs at eyed stage with povidone-iodine at concentrations of 50-1,000 ppm for 15-120 min

Table 1. The numbers of live *Flavobacterium psychrophilum* and *Aeromonas salmonicida* cells recovered from coho salmon eggs exposed to these bacterial suspensions

Bacteria	Eggs	Infection dose (CFU/mL)	Group	Experiment	Days after exposure			
					7	14	21	28
F. psychrophilum	Fertilized egg (before water-hardening)	1.0×10^8	Infected	I	1.3×10^6 *1	7.7×10^5	1.6×10^4	3.2×10^5
				II	2.4×10^5	8.0×10^5	3.3×10^5	2.6×10^6
		1.0×10^6	Infected	I	- *2	-	-	-
				II	-	-	-	-
		1.0×10^4	Infected	I	-	-	-	-
				II	-	-	-	-
		0	Uninfected	I	-	-	-	-
				II	-	-	-	-
	Fertilized egg (after water-hardening)	1.0×10^8	Infected	I	-	-	-	-
				II	-	-	-	-
		0	Uninfected	I	-	-	-	-
				II	-	-	-	-
	Eyed egg	1.0×10^8	Infected	I	-	-	-	-
				II	-	-	-	-
		0	Uninfected	I	-	-	-	-
				II	-	-	-	-
A. salmonicida	Fertilized egg (before water-hardening)	7.1×10^8	Infected	I	-	-	-	-
				II	-	-	-	-
		0	Uninfected	I	-	-	-	-
				II	-	-	-	-

*1 Bacterial numbers were expressed in CFU/g.

*2 Not detected. The lower limit of detection was 4×10^3CFU/g.

resulted in no reduction in the number of accompanied *F. psychrophilum*, despite the fact that the povidone-iodine solutions killed free *F. psychrophilum* completely by exposure to 20 ppm for 1.0 min or 40 ppm for 0.5 min. The viable counts of *F. psychrophilum* ranged from 10^4-10^6 CFU/g. Povidone-iodine concentration and the exposure time also had nothing to do with the number of live *F. psychrophilum* cells recovered from the surface-disinfected eggs. The povidone-iodine treatment is ineffective to eliminate *F. psychrophilum* from salmonid eggs. These results indicate that *F. psychrophilum* may be located at the inner part of the eggs where povidone-iodine cannot reach.

THE INVATION OF *F. PSYCHROPHILUM* INTO EGGS BY EXPERIMENTAL INFECTION

Although egg disinfection procedures using an organic iodine compound had been recommended, it was noted that iodophore treatment of eggs did not always prevent cold-water disease in subsequent fry (Holt *et al.*, 1993). Brown *et al.* (1997) showed evidence that *F. psychrophilum* was transmitted within salmonid eggs by cultivating naturally infected eggs. Thus, the ineffectiveness of povidone-iodine treatment against *F. psychrophilum* seems due to the invation of the pathogen into the salmonid eggs. Kumagai *et al.* (2000) attempted to determine whether *F. psychrophilum* enter eggs by experimental infection. The contents of the surface-disinfected eggs were aseptically removed using a syringe. *F. psychrophilum* was isolated from the egg contents after 7-50 days of incubation and the viable bacterial counts ranged from 10^3 to 10^7 CFU/g. The bacterium was also isolated from the chorion which had not been disinfected with povidone-iodine, whereas, no bacterium was isolated from the disinfected chorion (Table 2). Although a large number of the bacteria was recovered from the non-disinfected chorion which had been aseptically separated from experimentally infected eggs, no bacteria could be isolated from the chorion when it had been disinfected with povidone-iodine. This result indicated that disinfection with povidone-iodine completely killed *F. psychrophilum* colonizing on the egg surface. On the other hand, it was revealed that the contents separated from the infected eggs contained *F. psychrophilum* and that many *F. psychrophilum* cells were detected within the eggs by indirect fluorescent antibody technique in frozen sections of the infected eggs. These results indicate that *F. psychrophilum* is located at the inner part of the egg where povidone-iodine could not reach.

Since 1994, Researchers have recomended that fish farmers introduce domestic eggs into their hatcheries instead of imported eggs, and the prevalence of cold-water disease has steadily decreased.

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Table 2. The numbers of live *Flavobacterium psychrophilum* cells recovered from the content and chorion of coho salmon eggs exposed to the bacterial suspensions

Group	Site	Treatment	Experiment	Days after exposure			
				7	14	21	50
Infected	Chorion	No disinfection	I	9.4×10^4 *1	7.0×10^5	2.6×10^5	1.1×10^7
			II	4.0×10^4	3.2×10^7	-*2	2.8×10^5
	Chorion	Disinfection	I	-	-	-	-
			II	-	-	-	-
	Content	No disinfection	I	8.0×10^3	6.7×10^6	4.0×10^3	2.2×10^7
			II	4.0×10^4	2.9×10^5	1.6×10^4	1.8×10^6
Uninfected	Chorion	No disinfection	I	-	-	-	-
			II	-	-	-	-
	Content	No disinfection	I	-	-	-	-
			II	-	-	-	-

*1 Bacterial numbers were expressed in CFU/g.

*2 Not detected. The lower limit of detection was 4×10^3CFU/g.

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DEVELOPMENT OF VACCINE AGAINST COLDWATER DISEASE IN AYU, *PLECOGLOSSUS ALTIVELIS*

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ABSTRACT

Flavobacterim psychrophilum is the etiological agent of coldwater disease causing serious losses in ayu farming as well as in natural populations of ayu *Plecoglossus altivelis*, one of the most important freshwater fishes in Japan for food and also for recreational angling. However, no commercial vaccine has been available for the prevention of coldwater disease in ayu. Thus, we investigated the potency of oil adjuvants, Montanidae ISA-206, 264, 266, 763A, and a squalene emulsion (Squalene), to enhance the response of ayu to a formalin-killed bacterin (FKB) made from the pathogen. Ayu were challenged 4wk after vaccination by an intramuscular injection with live pathogenic *F. psychrophilum*. Mortalities of fish injected with FKB combined with any of the adjuvants showed that the adjuvanted vaccines had significantly higher ($P<0.05$) potencies than the FKB vaccine alone as well as the fish groups injected with sterile distilled water. Moreover, adjuvanted vaccines produced significantly higher antibody titers than the FKB vaccine without adjuvants. However, adjuvants Montanidae ISA-266, 264 and 206 are highly toxic in ayu, whereas adjuvant Montanidae ISA-763A shows low toxicity and handmade adjuvant Squalene did not show any toxicity in ayu. On the basis of efficacy and safety, it can be concluded that the 763A and Squalene vaccines are the most suitable for the prevention of coldwater disease in ayu.

Key words: oil adjuvant, squalene, bacterin, *F. psychrophilum*, vaccine, coldwater disease, ayu, *Plecoglossus altivelis*

INTRODUCTION

Flavobacterium psychrophilum was originally isolated from coho salmon *Oncorhynchus kisutch* in USA in 1948, as the causative agent of 'low temperature disease' or 'peduncle disease,' and later was named 'coldwater disease' by Wood and Yasutake in 1956, and this term is now the most widely used. Coldwater disease causes serious losses in juvenile rainbow trout in farming and also in wild populations throughout Europe and North America (Bernardet, 1997). Since 1980, a disease known as rainbow trout fry syndrome (RTFS) due to this bacterium has resulted in serious losses in rainbow trout hatcheries throughout Europe (Lorenzen and Olesen. 1997). This disease is severe not only in salmonid fish but also in many non-salmonid fish in many countries of the world (Lehmann *et al.*, 1991). In Japan, this pathogen was first isolated from ayu in 1987, and has spread throughout Japan (Wakabayashi *et al.*, 1994). Ayu is one of the most important freshwater fish in Japan for food and recreational angling. Recently, bacterial coldwater disease has caused high mortality in ayu farms and natural populations and is considered to be one of the most serious fish diseases in aquaculture in Japan. However, no commercial vaccines are currently available for the encounter of coldwater disease in ayu, although a whole cell inactivated vaccine has been reported against coldwater disease in trout (Obach and Baudin Laurencin, 1991). Therapeutic treatments are limited for the prevention of coldwater disease in ayu due to resistance of this bacterium to drugs. To mitigate the severity of losses, an effective vaccine is highly desire.

First, we tried to develop a vaccine using a formalin-killed bacterin (FKB), but encountered low efficacy. It has been well recognized that adjutants have become important tools for increasing vaccine potency to protect fish from bacterial disease. Adjuvants have been approved for use in fish vaccines in USA and Norway (Anderson, 1997; Markestad and Grave, 1997) with several injectible

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bacterins for *A. salmonicida* and the combinations of *A. salmonicida, Y. ruckeri* and *Vibrio* sp. by incorporating light oil into these commercial bacterins. The function of oil adjuvants as a reservoir of antigen or the mechanism of oil adjuvants is the depot effect.

Midtlyng *et al.* (1996) reported that adjuvants are very useful for the preparation of fish vaccine but cause several side effects. In a previous study (Rahman *et al.*, 2000), we found that the oil adjuvants Montanidae ISA-763A and handmade Squalene with FKB are very effective against coldwater disease in ayu. However, the toxicity of those oil adjuvants have not been investigated in ayu. In the present study, we tried to find a suitable vaccine against coldwater disease in ayu using a series of commercial adjuvants, such as Montanidae ISA and Squalane combined with an FKB of *F. psychrophilum*, by screening them prior to their efficacy and safety in ayu.

MATERIALS AND METHODS

Fish

Juvenile ayu *Plecoglossus altivelis* were obtained from a hatchery (Nissin Marine Tech) in Aichi Prefecture, Japan, where occurrence of coldwater disease has not been observed. The fish were confirmed to be free of *F. psychrophilum* before starting the experiments. The fish were maintained in 200-L tanks with running freshwater at 15℃, fed commercial dry pellets, and acclimatized to the laboratory for at least 4 wk before starting each experiment.

Bacteria

Flavobacterium psychrophilum strain PH-9304, originally isolated in 1993 from an infected ayu in Hiroshima Prefecture, Japan, was used in this study. Bacteria were cultured on modified cytophaga agar (MCY agar), pH 7.2 for 2 days (Wakabayashi and Egusa, 1974), and on casiton gelatin yeast agar (CGY agar, pH 7.2), for 1 day. (Ototake and Wakabayashi, 1985).

Adjuvants

Adjuvants used in this study are shown in Table 4.

Vaccine formulation

FKB vaccine: FKB was prepared by suspension of harvested bacteria in sterile distilled water at the concentration of 10^9 cells/mL, followed by addition of formalin to a final concentration of 0.3%.

763A vaccine: The commercial adjuvant Montanidae ISA-763A (Seppic, France) was combined with the FKB vaccine in a 7: 3 (v/v) ratio. The adjuvant was mixed with FKB by passing the mixture through a double-ended needle from one syringe to another repeatedly, until it became a thick homogenous emulsion.

266, 264 and 206 vaccines: The commercial adjuvants Montanidae ISA-266, 264 and 206 (Seppic, France) were combined with the FKB vaccine in a 1 : 1 (v/v) ratio to make a homogenous thick emulsion as mentioned above.

Squalene vaccine: The Squalene adjuvant was composed of a homogenous mixture of 7.5 mL canola oil (Sigma), 1 mL squalene (Wako, Japan) and 1.3 mL arlacel-A® (Nacalai Tesque, Japan). We prepared one mixture consisting of the canola oil, squalene and arlacel-A® and another mixture consisting of 10 mL FKB vaccine (10^9 CFU/mL) with 0.2 mL Tween 40 (Nacalai Tesque, Japan). Subsequently, the two mixtures mixed very well by using a double-ended needle. This emulsion was used as the Squalene vaccine.

Vaccination

The potency of each of the vaccines was assessed by intraperitoneal injection of 1.5 ± 0.5 g (average body weight ± standard deviation) juvenile ayu. One hundred ayu of each group were vaccinated with 10 μL per fish of the 763A, 266, 264, 206, the Squalene or the FKB vaccines, and another group was injected with sterile distilled water as a control. Injections were carried out according to our previous study (Rahman *et al.*, 2000) using a repeating micro dispenser (Eppendorf 4780) with a 28-ga, 4-mm-long needle. A polypropylene tube was attached to the needle so that it would not enter the fish deeper than 1.5 mm.

Challenge test

To determine the efficacy of vaccines, challenge tests were performed 4 wk after vaccination according to our previous study (Rahman *et al.*, 2000). Fish were anaesthetized with 0.015% FA-100 (4-allyl-2-methoxyphenol, Tanabe Seiyaku Co., Japan) and then challenged by intramuscular injection with 25 μL of a live *F. psychrophilum* suspension at concentrations of 1.5×10^4 and 6×10^4 CFU/fish as high doses and 1.5×10^3 and 6×10^3 CFU/fish as low doses. Challenged fish were kept in tanks with running freshwater at 15℃ for 15 days and mortality was recorded daily. Infection by *F. psychrophilum* was confirmed from the symptom of the coldwater disease in dead fish and also from morphological characteristics of the colony of bacteria isolated from the kidney of dead fish. Relative percentage survival (RPS) (Croy and Amend, 1977) was calculated from the cumulative mortalities as:

$$RPS = \left(1 - \frac{\textit{mortality of vaccinated group}}{\textit{mortality of unvaccinated control group}}\right) \times 100.$$

Agglutination antibody titer

Serum was collected by caudal puncture from 15 fish in each group 4 wk after immunization. Sera were incubated at 44 ℃ for 20 min to inactivate complement activity (Sakai, 1981). The agglutinating antibody titer against *F. psychrophilum* was determined using the micro titer method.

Toxicity of adjuvants in l.c. Juvenile ayu

To determine the toxicity of adjuvants, intraperitoneal injection was carried out in ayu with 10, 25 and 50 μL of each adjuvant in 3 groups of ayu each consisting of 8 fish and control groups were injected with distilled water. Injected fish were kept at 15℃ for 3 wk and mortality of ayu was recorded daily. Finally, the toxicity of adjuvants was determined from the mortality of ayu.

Statistical analysis

Significant differences between treatments in levels of mortality were detected by Fisher's exact probability test. The relative percentage survival was calculated when a significant difference was detected between mortalities in the control and vaccinated groups. Data for the agglutination antibody titer were first subjected to the F test in order to detect significant differences between variances. When the difference of variances was significant, the data were subjected to the Cochran-Cox test in order to detect significant differences between means. The rest of the data were subjected to the Student's unpaired t test in order to detect significant differences between means. The significance level for all statistical analyses was $P<0.05$.

RESULTS

In the artificial challenge of ayu with live pathogens at a high dose of 1.5×10^4 CFU/fish, the accumulated mortality showed 24% in the Squalene vaccinated group, 30% in the 763A vaccinated group and 45% in the FKB vaccinated group, whereas mortality in the control group injected with sterile distilled water showed 75%. The challenge with low dose did not perform countable mortality in the control fish group the including the vaccinated group. And in the other challenge of ayu with a high dose of 6×10^4 CFU/fish, accumulated mortality showed 28% in the 266 vaccinated group, 64% in the 264 vaccinated group, 40% in the 206 vaccinated group, 68% in the FKB vaccinated group and 88% in the control group injected with sterile distilled water. In the challenge with a low dose of 6×10^3 CFU/fish, accumulated mortality showed 18% in the 266 vaccinated group, 10% in the 264 vaccinated group, 15% in the 206 vaccinated group, 35% in the FKB vaccinated group and 60% in the control group. The relative percentage survival (RPS) of groups administered with the adjuvanted vaccines as higher than at administered with the FKB vaccine alone except in the 264 vaccinated group in the challenge with high dose, while the FKB vaccine showed higher RPS than the control (Table 1).

Table 1. Relative percentage survival (RPS) of vaccinated ayu after challenge with *F. psychrophilum*

Vaccines	Challenge dose (CFU/fish)	Total (fish)	Specific loss (fish)	Mortality (%)	RPS (%)
Squalene	1.5×10^4	25	6	24 *	68
	1.5×10^3	25	2	8	
763A	1.5×10^4	20	6	30 *	60
	1.5×10^3	20	2	10	
FKB	1.5×10^4	20	9	45	40
	1.5×10^3	20	2	10	
Control	1.5×10^4	20	15	75	
	1.5×10^3	20	2	10	
266	6×10^4	25	7	28 *	68
	6×10^3	12	2	18 *	70
264	6×10^4	25	16	64 *	27
	6×10^3	20	2	10 *	83
206	6×10^4	25	10	40 *	54
	6×10^3	20	3	15 *	75
FKB	6×10^4	25	17	68	23
	6×10^3	20	7	35	41
Control	6×10^4	25	22	88	
	6×10^3	20	12	60	

* significantly ($P<0.05$) lower than control.

The agglutinating antibody titers of sera from fish in the vaccinated, non-vaccinated and pre-immunized groups are shown in Table 2. The highest antibody titers were found in the 763A vaccinated group followed by the 266, 206, Squalene and 264 vaccinated groups. The FKB vaccinated fish showed higher antibody titers than control fish, which showed antibody titers similar to those of pre-immune fish. The antibody titer of the adjuvanted vaccines vaccinated group is significantly higher than the FKB vaccinated group or control group. However, there is no significant difference in titer among the adjuvanted vaccine vaccinated group.

The toxicity of oil adjuvants is shown in Table 3. Oil adjuvants ISA-266, 264 and 206 showed high mortality of ayu by intraperitoneal injection at 50 μL/fish and even 25 μL/fish. On the other hand, there was no mortality of ayu injected with 50 μL/fish of Squalene. The ISA-763 showed also very low mortality after injecting 50 μL/ayu.

Table 2. Agglutinating antibody titers in sera of vaccinated and non-vaccinated ayu after 4 wk vaccination

Vaccine	Agglutinating antibody titer (Log_2)										
	Individually										Av.±SD
Squalene	5,	5,	4,	2,	4,	4,	5,	5,	4,	5	4.3±0.7 *a
763A	6,	5,	4,	5,	4,	5,	4,	4,	6,	5	4.8±0.6[a]
ISA 266	5,	4,	5,	5,	3,	5,	5				4.6±0.6[a]
ISA 264	6,	5,	5,	4,	3,	3,	4				4.3±0.8[a]
ISA 206	4,	5,	5,	3,	4,	5,	5				4.4±0.7[a]
FKB	1,	3,	1,	1,	1,	2,	1,	2,	2,	2	1.6±0.6[b]
Control	<1,	<1,	<1,	<1,	<1,	<1,	<1,	<1,	<1,	<1	<1[c]
Pre-immunized fish	<1,	<1,	<1,	<1,	<1,	<1,	<1				<1[c]

* Geometric mean reciprocal $\log_2$ value of the highest dilution rate of the serum which showed positive agglutination.
*, significant differences ($P < 0.05$) among a, b, c.

Table 3. Toxicity of oil adjuvants in juvenile ayu by intraperitoneal injection

Adjuvant	Mortality Injected fish/dead fish and % of mortality					
	Dose/fish	10 μl		25 μl		50 μl
		(Injected fish/Dead fish (% mortality))				
Squalene		8/0	(0%)	8/0	(0%)	8/0 (0%)
ISA-763		8/0	(0%)	8/0	(0%)	8/1 (13%)
ISA-266		8/0	(0%)	8/5	(63%) *	8/8 (100%) *
ISA-264		8/0	(0%)	8/5	(63%) *	8/7 (88%) *
ISA-206		8/0	(0%)	8/3	(38%)	8/6 (75%) *
DW		8/0	(0%)	8/0	(0%)	8/0

*, significantly ($P<0.05$) higher than control (DW)

DISCUSSION

In this study, we found that the adjuvants ISA-266, 264 or 206 are acutely toxic in ayu. However, all adjuvants with FKB induced high antibody titer and a high level of protection against coldwater disease in ayu. We found an exception in the 264 vaccinated group in that challenge with a high dose did not provide high protection although it induced high antibody titer; however, the challenge with a low dose provided higher protection. In the challenge with a high dose, the reason for low protection in the 264 vaccinated group is unknown. The intraperitoneal injection with 50 μL of the oil adjuvant Squalene adjuvant/1.5 g fish, show a no toxicity and Montanidae ISA-763A showed minor toxicity in ayu. Moreover, these two adjuvants with FKB are very effective in inducing antibody titers with a high level of protection. We have conducted several challenge tests to further investigate the efficacy of the Squalene and the 763A vaccines and found similar results to those shown in Table 1. However, we did not perform several challenge tests to investigate the efficacy of the 266, 264 and 206 vaccines due to their toxicity in ayu. On the basis of efficacy and safety (Table 4), it is concluded that the Squalene and the 763A are the most suitable vaccines against coldwater disease in ayu which may encounter a serious problem in aquaculture in Japan.

This result agrees with the previous study reported by Anderson (1997) who showed that the adjuvant Titermax (produced by CytRx Corp, Norcross, Georgia, USA) was very effective in inducing protection and high antibody titers in salmon, but granulomas were severe and present in all visceral organs. Midtlyng and Lillehaug (1998) reported that intraperitoneal administrations of vaccines containing oil-based adjuvants could adversely affect the growth of Atlantic salmon. Macy (1997) also reported that the use of adjuvants in veterinary vaccines have caused numerous side effects. In this study, however, we did not find any chronic side effects such as granulomas or abscess formations in ayu administered with the oil adjuvants combined with FKB. Furthermore, the growth of treated ayu was similar to that of control groups. This result agrees with a previous study by Leenaars *et al.* (1998) who reported that subcutaneous injection with the ISA-50 adjuvant in mice induced acceptable antibody titers with little side effects. We also found that the

Table 4. Suitable vaccine against coldwater disease in ayu

Vaccine	Emulsion type	Type of oil	Adjuvant and antigen ratio	Efficacy	Safety	Status
FKB-	-	-	-	×	○	×
763A	W/O	Non-mineral oil	7 : 3	○	○	○
Squalene	O/W +Shark oil	Plant seed oil	1 : 1	○	○	○
ISA-266	W/O/W and non-mineral oil	A mixture of mineral	1 : 1	○	×	●
ISA-264	W/O/W	Non-mineral oil	1 : 1	×	×	×
ISA-206	O/W	Mineral oil	1 : 1	●	×	×

* W/O: water in oil; W/O/W: water in oil in water; O/W: oil in water; × : not effective or not safe or not useful as a vaccine; ○ : very effective or safe or very good vaccine; ● : moderately effective or not good vaccine.

retention of adjuvants Squalene and ISA-763 in ayu is less than 8 wk (data not shown).

In our previous study (Rahman *et al.* 2000), we found high agglutinating antibody titers in the sera of juvenile ayu 4 wk after injection with the oil adjuvants Squalene or Montanidae ISA 763A combined with FKB of *F. psychrophilum*. In the present study, we also found that the agglutinating antibody titer was higher in sera of fish injected with vaccines containing the Squalene, 763A, 266, 264 and 206 adjuvants than in fish injected with sterile distilled water or the vaccine containing no adjuvant. This result suggests that an oil adjuvant enhances the humoral immunity of ayu as well as salmonids. In the case of the ISA 264 adjuvant, we found high agglutinating antibody titers with a low level of protection in the challenge with a high dose of the pathogen. However, the challenge with a low dose demonstrated very good protection.

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POTENTIAL IMPACT OF *RENIBACTERIUM SALMONINARUM* ON ADULT RETURNS OF SNAKE RIVER SPRING/SUMMER CHINOOK SALMON

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ABSTRACT

Snake River spring/summer chinook salmon populations declined rapidly during the mid- to late 1970s after construction of four lower Snake River dams. Considerable efforts followed to improve the direct survival of downstream-migrating juvenile smolts through these four dams, and through four additional dams on the lower Columbia River. Presently, although survival of downstream migrants is now as high or higher than it was prior to construction of the Snake River dams, adult return rates remain low. Therefore, other factors that have increased mortality compared to pre-dam conditions must act on the fish in the lower Columbia River estuary or in the ocean. One hypothesis is that *Renibacterium salmoninarum*, a bacterium found in nearly all Snake River chinook salmon and the causative agent of bacterial kidney disease (BKD), is responsible for decreased adult returns. This hypothesis assumes that the disease is triggered by stress associated with bypass systems at dams or during the smolt transportation process. In a related hypothesis, hatchery fish with high levels of *R. salmoninarum* intermingle with wild fish and spread infections, which result in increased mortality. A final hypothesis is that decreased ocean productivity may account for the low chinook salmon returns.

Key words: chinook salmon, disease, survival, Snake River

The Columbia River watershed historically produced more chinook salmon (*Oncorhynchus tshawytscha*) than any other river system in the world (Netboy, 1980), with the majority of spring chinook salmon originating in the Snake River basin (Fulton, 1968) (Figure 1). In the early 1880s, spring and summer chinook salmon provided commercial fisheries in the lower Columbia River with average annual catches of 17.7 million kg (Craig and Hacker, 1940). Heavy exploitation by these fisheries, however, caused a substantial depletion of the dominant summer stock; the fisheries then concentrated on the spring and fall stocks (Craig and Hacker, 1940; Gangmark, 1957). Summer chinook salmon populations from the mid- and upper Columbia River continued to decline such that by 1964, the inriver commercial fishery for all summer fish was closed. By this time, Snake River basin spring/summer chinook salmon accounted for approximately 78% of the remaining upper river populations (Fulton, 1968).

In addition to overfishing, further causes of stock declines in the early years were habitat destruction and damming of tributaries for water withdrawal and small-scale hydropower (Craig and Hacker, 1940). Populations of wild Snake River spring/summer chinook salmon continued to decrease coincident with construction of hydroelectric dams on the lower Snake and Columbia rivers (Raymond, 1988; Williams, 1989). Smolt-to-adult return rates of these populations fell from greater than 4% in the mid- to late 1960s, when only four dams were in place, to generally less than 2% during the 1970s after seven or eight dams were in operation (Figure 2). This large additional decrease in adult return rates coincided with substantially reduced juvenile survival (Raymond, 1979) with concurrent high levels of descaling and injury to fish (Williams and Matthews, 1995). As a consequence of low returns, harvest rates of 40-60% were reduced to less than 10%.

To address the low adult returns, two major directions were pursued: (1) changes to hydropower system operation

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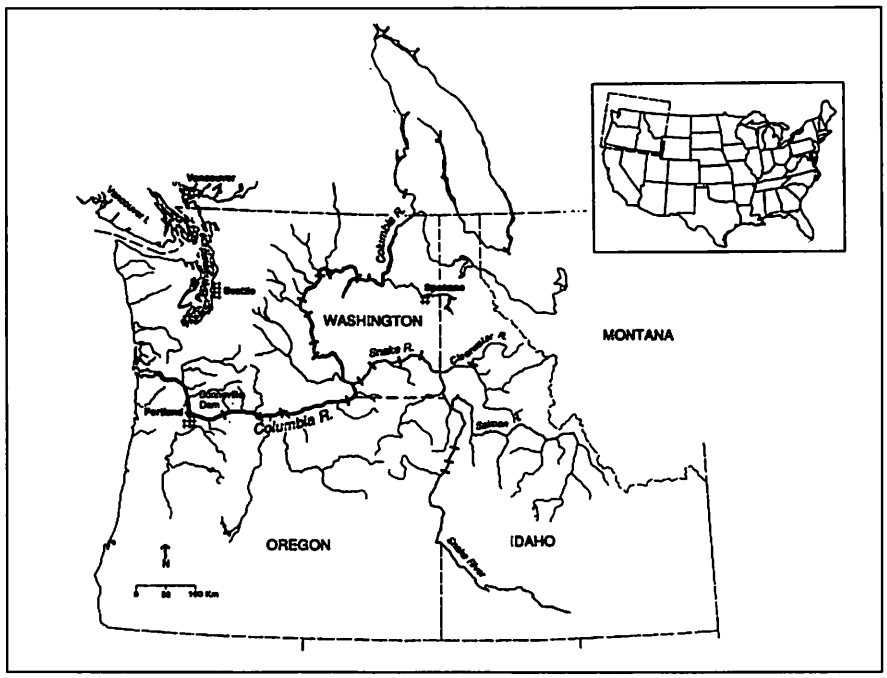

Figure 1. Map of the Columbia River basin.

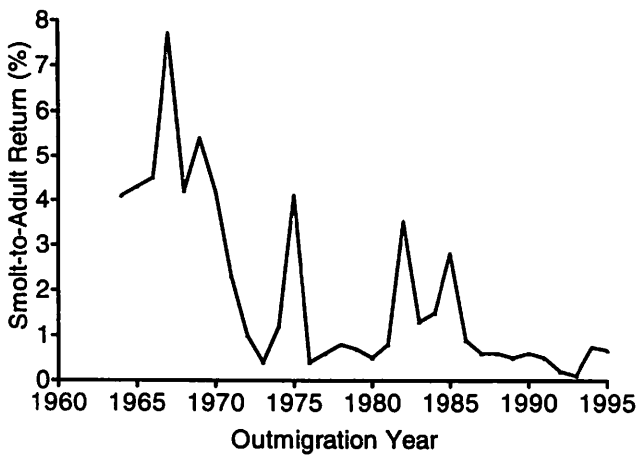

Figure 2. Smolt to adult returns of wild Snake River spring/summer chinook salmon.

and the construction of fish-handling and bypass structures at dams to increase survival of juvenile migrant fish, and (2) construction of fish hatcheries to increase smolt production. Major changes in hydropower system operation included increases in river flow, removal of debris from forebays at dams, more efficient and continuous turbine operation, and installation of "flip-lips" on spillways to decrease atmospheric gas supersaturation. Considerable resources were invested in the construction of facilities to bypass juvenile fish away from turbines, and this construction was followed by extensive evaluation and modifications to bypass facilities that were found inadequate (Williams, 1989; Williams and Matthews, 1995). Further, barges were constructed to transport fish collected at upper dams to a release site below Bonneville Dam. As a result of these efforts, survival of juvenile fish that migrate downstream through the hydropower system is now as high as it was prior to construction of the dams (Figure 3) (Williams *et al.*, 2001) (Figure 3), and direct survival of fish transported around the hydropower system is nearly 100%. Additionally, Schreck and Stahl (1998) found that survival was greater than 90% for fish migrating between the tailrace of Bonneville Dam to near the mouth of the Columbia River. Surprisingly, these substantial improvements in downstream migrant smolt survival have not led to concurrent increases in adult return rates of chinook salmon.

The continued low adult return rates, but relatively high freshwater smolt survival, indicate that factor (s) limiting survival to adulthood are those that act on fish during ocean residence. Tremendous controversy exists over the possible factor (s) and the mechanism (s) that limit adult returns (Schaller *et al.*, 1999; Anderson, 2000; Zabel and Williams, 2000). The following different factors are hypothesized to have caused increased ocean mortality: (1) increased rates of bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* infections contracted during hydropower system passage, or exacerbated by stress from passage; (2) hatchery overproduction which has increased competition for food, stress levels, or transmission of *R. salmoninarum*; and (3) changed ocean conditions leading to changes in predator/prey dynamics. However, empirical evidence to support these hypotheses is lacking.

In the late 1980s, Raymond (1988) and Williams (1989)

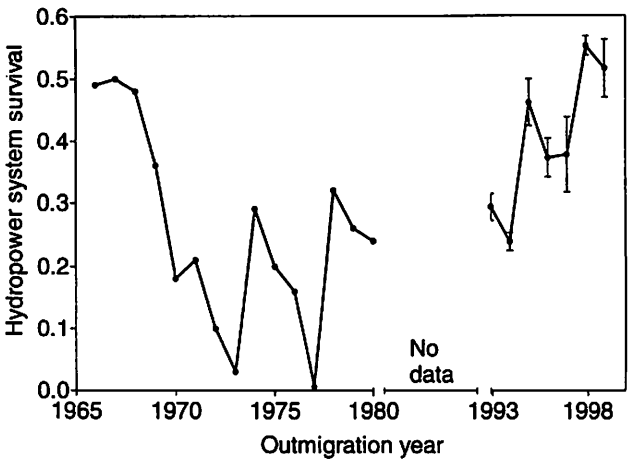

Figure 3. Estimated survival of juvenile Snake River spring/summer chinook salmon through the lower Snake and lower Columbia river hydropower system. Four dams were in place in 1966-67, five in 1968, six in 1969, seven in 1970-74, and eight from 1975 to present.

hypothesized that a likely cause of decreased adult returns was an increased loss of fish from BKD. Bacterial kidney disease is a chronic, systemic disease present in salmonid stocks from virtually every river basin draining to the Northeast Pacific Ocean and is considered more severe in spring/summer chinook salmon than in other salmonids (Bullock and Wolf, 1986). Kent *et al.* (1998) in a survey of salmonid pathogens in catch from ocean fisheries in British Columbia found that 44 of 77 chinook salmon tested positive for *R. salmoninarum*, and this infection rate was substantially greater than that of any other salmonid species.

Most Snake River chinook salmon harbor *R. salmoninarum*. Using the enzyme immunosorbent assay (ELISA), Maule *et al.* (1996) found an *R. salmoninarum* infection rate of nearly 100% at some Snake River chinook salmon hatcheries in 1988, with a mean infection rate of 68% measured between 1988 and 1992. In contrast, the mean infection rate of migrant fish monitored at Lower Granite Dam was 91%, suggesting that either *R. salmoninarum* infections progressed during migration, or that wild fish with higher infection levels increased the average infection rate in samples taken at Lower Granite Dam. From 1988-1991, Elliot *et al.* (1997) sampled migrant smolts at Lower Granite Dam and, based on ELISA techniques, determined that 86-100% of the fish were infected with *R. salmoninarum*, with a 100% infection rate in all wild fish samples. Although a high percentage of the fish were infected, the infection levels were considered low. Vanderkooi and Maule (1999) continued hatchery monitoring from 1993-1996 and found that *R. salmoninarum* infections fluctuated between high and low levels, but levels of infections were considered low in greater than 90% of the fish sampled.

Although *R. salmoninarum* infection rates are high in chinook salmon throughout the Columbia River basin, clinical BKD does not necessarily follow. Bakke and Harris (1998) suggested *R. salmoninarum* exists normally in the gut of all wild and hatchery Atlantic salmon, but at times of stress migrates to the kidneys and causes BKD. In chinook salmon, increases in stress are presumptively necessary for most cases of BKD to occur, as susceptibility to disease in fish increases under stressful conditions (Maule *et al.*, 1989; Sunyer *et al.*, 1995; Bakke and Harris, 1998; Van Muiswinkel *et al.*, 1999).

Yearling chinook salmon smolts from the Snake River experience elevated levels of stress, as measured by blood chemistry, when they are routed through juvenile bypass systems or loaded into barges for transportation (Bjornn *et al.*, 1985; Bjornn and Congleton, 1987). Some of the increased stress may result from passing through flumes or pipes in small volumes of water. Wild fish may experience elevated stress levels from interactions with high concentrations of larger hatchery fish. To evaluate the potential impacts of stress on the longterm survival of yearling chinook salmon, Park *et al.* (1986) conducted an extended seawater holding experiment in 1985. They sampled juveniles from several areas in a juvenile bypass system and from barges after loading, subsequently holding the fish for 141 days. Subsamples from the groups indicated a range of stress response. The major cause of mortality (range 60-75% from the least to the most stressed groups, respectively) was BKD. High levels of *R. salmoninarum* were found in all of the dead fish, but survivors had relatively low levels of infection.

In addition to experiencing direct mortality, Mesa *et al.* (1998) found that fish experimentally infected with *R. salmoninarum* were more susceptible to predation. In the latter stages of BKD, Mesa *et al.* (1999) observed higher levels of stress (measured through blood chemistry) which they attributed to the disease itself. They also suggested that the smoltification process may have triggered BKD. In both instances, they caution that their results were derived from laboratory experiments and may not apply in the wild. Further, BKD infections acquired in freshwater may impair the ability of salmonid smolts to acclimate to seawater, and entry into seawater may actually accelerate mortality among infected fish (Fryer and Sanders, 1981; Banner *et al.*, 1983; Moles, 1997).

R. salmoninarum is slow-acting and, therefore, mortalities due to BKD are likely to occur after fish reach the ocean. Banner *et al.* (1983) found that for chinook salmon, the percentage of mortality attributable to BKD increased the longer fish were held in seawater tanks. Spring chinook salmon held 200 days had mortalities ranging from 45-81%. Thus, it is not surprising that few mortalities during downstream migration are attributable to BKD. Further, since most chinook salmon juveniles (wild and hatchery) apparently harbor *R. salmoninarum* and the disease is transmitted both vertically and horizontally, removal of

the organism from the population appears unlikely. In fact, past attempts to treat or control hatchery infection levels through the use of antibiotics may have caused more harm than good, as *R. salmoninarum* is found intracellularly and antibiotics only inhibit its growth extracellularly (Bullock and Wolf, 1986). Keeping fish alive in the hatchery with antibiotics may not decrease subsequent BKD-related mortality. On the contrary, antibiotics may allow highly infected fish to live, at least temporarily after release from the hatchery, which may allow them to shed more bacterium and increase the probability of keeping a high disease incidence in the population.

A better solution appears related to segregation of adult spawners by disease level. Maule *et al.* (1996) attributed the decreased infection rates at hatcheries between 1988-1992 to hatchery practices that culled eggs from adult female spawners with high *R. salmoninarum* infections, segregation of eggs by severity of infection for the remainder of the brood, and reduced rearing densities. Segregation of eggs by severity of infection was implemented based on research by Elliot *et al.* (1995). Elliot *et al.* (1995) held juvenile spring chinook salmon in seawater net pens for 98 days and found a 12% mortality for fish with low infection levels (based on optical densities and ELISA techniques) and a 44% mortality for fish with high infection levels. Nearly 85% of fish in the high infection group were determined to have *R. salmoninarum* infections based on FAT.

Since most Snake River chinook salmon likely carry *R. salmoninarum* and experience increased levels of stress during their downstream migration, this increased stress may decrease immune system response. Thus, the hypothesis that BKD likely is responsible for decreased adult returns is viable. Nonetheless, direct empirical evidence linking hydropower-system passage to increased BKD mortality is lacking. But because wild Snake River chinook salmon stocks have not rebounded since changes were made to improve hydropower system passage, continued efforts are needed to address the problem. Eliminating all stressful conditions encountered by migrants within the hydropower system does not appear feasible as most fish will experience some stresses at bypass systems or during the transportation process. Further, periodic regime shifts in the ocean will continue to occur. The impact of hatchery fish on wild fish is unknown, but it is important to limit the extent to which disease transmission might play a role in decreased adult returns. It is unlikely that elimination of *R. salmoninarum* from hatchery or wild fish is possible. Thus, it is critical that hatcheries take all measures possible to eliminate releases of individual fish with high infection levels.

Data to resolve the controversy about causes for continued low chinook salmon returns will not likely become available soon. Meanwhile, it seems prudent to continue evaluating impacts of the hydropower system and following hatchery rearing practices that reduce horizontal transmission of *R. salmoninarum* to the greatest extent possible, both within hatcheries and after fish are released.

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STRESS IMPAIRS NON-SPECIFIC DEFENSE ACTIVITY OF FISH

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ABSTRACT

We investigated the effect of stress on the non-specific defense activity of fish. Because tilapia *Oreochromis niloticus* are aggressive, within a few hours of post-transferring two fish of different sizes into an aquarium, large fish always became dominant, and charged, chased and rammed subordinate small fish. The cortisol concentration in the plasma of the subordinate was significantly increased over that of the dominant, indicating that the subordinate was stressed. The number of neutrophils that migrated to the swim bladder, where formalin-killed bacterial cells were injected, was significantly decreased in the subordinate, and their phagocytic and respiratory burst activities were both reduced. A stress hormone, cortisol, also suppressed *in vitro* degranulation of tilapia eosinophilic granular cells, which are thought to contain neutrophil-migrating factor(s) in their granules. Artificial challenge with *Edwardsiella tarda* revealed the elevated susceptibility of the cortisol-implanted fish to edwardsiellosis. The results obtained from the present study suggest that secreted cortisol under stressful conditions directly impairs the non-specific cellular defense in tilapia, and therefore the stressed fish fail to defeat invading microorganisms.

Key words: stress, non-specific defense activity, tilapia, *Oreochromis niloticus*, neutrophil, eosinophilic granular cell, cortisol

INTRODUCTION

Some diseases appear to be triggered by stress. Bacteria gill disease, columnaris and furunculosis will be seen as a result of crowding, and bacterial kidney disease is affected by water hardness (Wedemeyer, 1997). There are many reports that stress induces alteration in immunocompetence. Stress reduces antibody production (Thompson *et al.*, 1993), lysozyme activity (Tort *et al.*, 1996), complement activity (Yin *et al.*, 1995) or natural killer (NK) cell activity (Ghoneum *et al.*, 1988), but enhances phagocytosis of neutrophils (Peters *et al.*, 1991) or number of granulocytes in peripheral blood (Cooper *et al.*, 1989).

Neutrophils are the first cells that migrate to the site of inflammation (Suzuki and Hibiya, 1988). Thus, pathogenic microorganisms have to escape from neutrophil defense function to establish infection. Stress could suppress the neutrophil function. The aim of this study is to investigate the effect of stress on the neutrophil defense function of fish.

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MATERIALS AND METHODS

Fish

Tilapia *Oreochromis niloticus* were used as experimental fish. Fish were maintained at 25℃ in 180-L tanks with a recirculating system and fed daily with dry pellets.

Cortisol measurement

Plasma cortisol concentration was measured by using commercial kits (Enzaplate cortisol, Chiba Corning Diagnostics Inc.).

Neutrophil preparation

Neutrophils were collected from the swim bladder according to the method of Endo *et al.* (1997). Briefly, fish were injected with formalin-killed *Escherichia coli* (1 mg/fish) into the swim bladder. At 24 h post-injection, neutrophils exuded into the swim bladder were harvested. The collected neutrophils were resuspended at a density of

1 x 10^6 cells/mL in Hanks balanced salt solution (HBSS) without magnesium, calcium and phenol red.

Phaogcytosis and respiratory burst of neutrophils

The neutrophil suspension was mixed with an equal volume of opsonized zymosan (0.5 mg/mL), and incubated with gentle rotation at 25℃ for 1 h, followed by being smeared onto glass slides. Phagocytosis of neutrophils was observed microscopically, and expressed as the index of phagocytosis (Le Morvan *et al.*, 1997).

Superoxide anion generated from neutropils was measured by CLA-dependent chemiluminescence according to Iida and Wakabayashi (1995). Phorbol 12-myristate 13-acetate (Sigma, USA) was used as a stimulator for neutrophils.

Administration of cortisol

Cortisol stock solution was prepared by the method of Tripp *et al.* (1987). The concentration of the stock solution was checked using the kit as described above. Cortisol implantation (5 mg/kg body weight) into the peritoneal cavity was done according to Pickering and Duston (1983). Implants were prepared by suspending cortisol in liquid coconut oil at 50℃ and injecting the warm liquid into the peritoneal cavity of the fish. Control fish were implanted with liquid coconut oil.

Determination of the degranulation of eosinophilic granular cells in vitro

Tissue-resident eosinophilic granular cells (EGCs) were taken from the swim bladder lumen by the method of Matsuyama and Iida (2000). The EGCs were suspended in cortisol solution containing substance P (200 μg/mL, Wako), which induces the degranulation of EGCs.

Edwardsiella tarda infection

Twenty fish were used for the each group. Cortisol or coconut oil-implanted fish were injected with *Edwardsiella tarda* FPC498 (1 mg/100 g body weight) into the swim bladder. Death by *E. tarda* was monitored for 2 wk after the injection. Two weeks after the injection, the infection of *E. tarda* was examined by reisolaiting the microorganism from kidneys of survival fish.

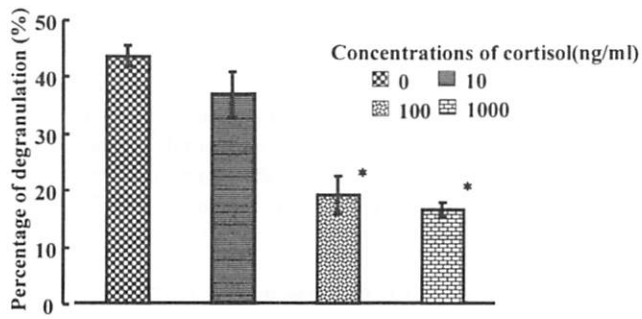

Figure 2. Effect of cortisol on degranulation of tilapia mast cells *in vitro* Substance P was used as a stimulator for degranulating tilapia mast cells Bars marked * are significantly different from control.

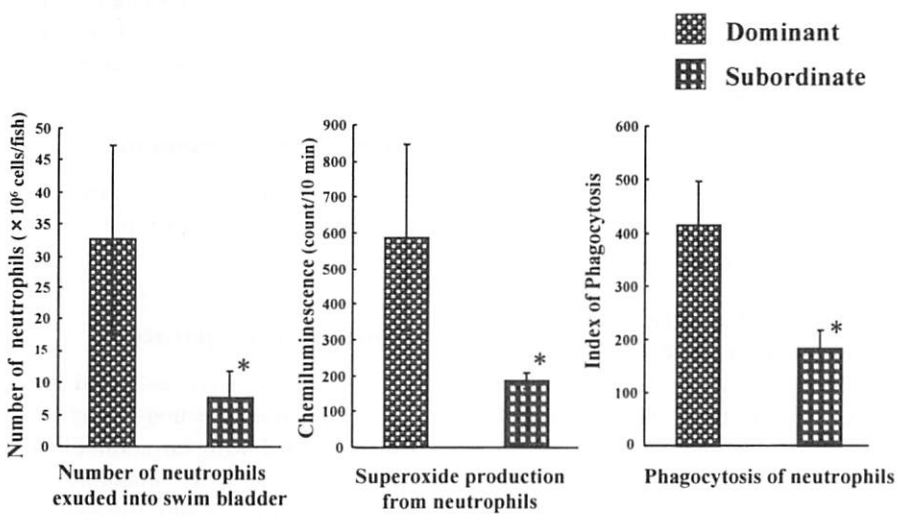

Figure 1. Neutrophil defense activities in dominant and subordinate fish Bars marked * are significantly different.

RESULTS

Plasma cortisol level

Within a few hours of post-paring with different sized fish, the large fish always bite, charged, chased and rammed their partners, indicating that the large fish were dominant. The cortisol level in plasma of the subordinates (20.0±3.9 ng/mL) was significantly higher than those of the dominants (14.0±2.7 ng/mL).

Phagocytosis and respiratory burst of neutrophils

The number of neutrophils collected from the subordinates was significantly smaller than that of the dominants. The neutrophils from the subordinates apparently suppressed phagocytosis and respiratory burst (Figure 1).

Effect of cortisol on the degranulation of EGCs in vitro

The percentage of EGC degranulation was lowered by cortisol dose dependently. Cortisol at concentrations of more than 100 ng/mL significantly reduced the ability of EGCs for degranulation (Figure 2).

Edwardsiella tarda infection

Four out of 20 fish implanted with cortisol were dead, while no dead fish were observed in the controls. Nine out of 16 survivals in the cortisol-implanted group were infected by *E. tarda* at 2 wk post-injection. However, *E. tarda* was recovered from only one control fish after the experiment.

DISCUSSION

In the present study, we demonstrated the adverse effect of social stress in tilapia on their neutrophil defense activities by phagocytic and respiratory burst activities. Higher plasma cortisol concentration in the subordinate fish was observed under the social stress. Because cortisol suppressed both the neutrophil activities *in vitro* (data not shown), we concluded that cortisol elevated under stressful conditions directly impairs the neutrophil activities.

The degranulation of tilapia EGCs is involved in the migration of neutrophils to the site of inflammation (Matsuyama and Iida, 1999), and the EGCs release neutrophil-migrating factor(s) (Matsuyama and Iida, 2000). In the present study, we observed less number of neutrophils exuded into the swim bladder of the subordinates, and cortisol inhibited the EGC degranulation. It is thought that the inhibited degranulation of the EGCs by cortisol is responsible for less exudation of the neutrophils to the site of inflammation in the stressed tilapia.

Neutrophils play an important role in host defense against the first stage of infection, with neutrophils being the first phagocytes appearing at the inflammatory site (Suzuki and Hibiya, 1988). Neutrophils with impaired defense functions by stress could not block infections. In fact, *E. tarda* was easy to establish infection in cortisol-implanted tilapia in the present study. Some diseases appear to be triggered by stress, such as columnaris, furunculosis and vibriosis (Wedemeyer, 1997), and stress reduces lysozyme activity (Tort *et al.*, 1996), complement activity (Yin *et al.*, 1995) and NK cell activity (Ghonem *et al.*, 1988). These impaired non-specific defense mechanisms, including the suppressed neutrophil functions demonstrated in the present study, could increase susceptibility to infections and allow the infections to spread in the stressed fish.

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EMACIATION DISEASE OF CULTURED TIGER PUFFER *TAKIFUGU RUBRIPES*

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ABSTRACT

A new disease characterized by serious emaciation has been spreading among cultured tiger puffer *Takifugu rubripes* in Japan. Hitherto unknown myxosporeans have been collected from the intestine of emaciated fish and described as *Myxidium fugu, Myxidium* sp. and *Leptotheca fugu*. Two hyperparasitic microsporeans were often found in the plasmodia of *M. fugu* and *L. fugu*, but their pathogenic effects to host myxosporeans remain to be clarified. These myxosporean infections had some exceptional features. First, they showed no clear seasonality in their development, and mature spores were rarely observed. Secondly, two of them (*Myxidium* sp. and *L. fugu*) are histozoic in the intestine of the host. Thirdly, they probably transmit directly from fish to fish. Histologically, *M. fugu* showed minimal pathogenicity. On the other hand, in *Myxidium* sp. infection, the epithelium was detached and dead cells accumulated between the epithelium and lamina propria. In *L. fugu* infection, infiltrated macrophages surrounded plasmodia, and resultant parasite-macrophage aggregates moved to the lamina propria to form macrophage centers. These host responses caused the basement membrane of the epithelium to be discontinuous, leading to epithelial decomposition. When *L. fugu* was infected with a hyperparasitic microsporean, these pathological changes tended to be more severe. It is evident histologically that *Myxidium* sp. and *L. fugu* with or without the hyperparasitic microsporean were highly pathogenic to the host fish, and this strongly suggests that they are the causative agents of the emaciation disease.

Key words: tiger puffer, *Takifugu rubripes*, Myxosporea, Microsporea, etiology, histopathology

INTRODUCTION

Culture of tiger puffer *Takifugu rubripes* started in the 1950s to catch wild fish in spring and maintain them until winter, when they are consumed. The annual culture production of tiger puffer in Japan stayed as low as less than 100 t until the end of the 1970s. It soared dramatically after the introduction of artificially produced puffer progeny for culture in the early 1980s. The production reached its peak of 5, 961 t in 1997. They are cultured in the western part of Japan, mainly in Kumamoto and Nagasaki Prefectures, Kyushu.

Most typically, tiger puffer seeds are introduced to culture sites in early summer, cultured in floating net cages in coastal areas and fed with pelleted feeds. They reach a commercial size of about 1 kg the following winter. The standard culture period is 1 and a half year.

Tiger puffer is not an easy species to culture, with many disease problems, especially those caused by parasites (Ogawa and Yokoyama, 1998). Since 1996, a hitherto unknown disease has appeared among cultured tiger puffer in Kyushu. Affected fish showed clinical signs of severe emaciation: sunken eyes, bony ridges on the head and a tapered body (Figure 1). Sometimes the incidence was so high that most of the stock was affected by the disease. The etiological agent (s) were unknown, but circumstantial evidence strongly suggested that this is a contagious disease.

INVESTIGATIONS OF THE ETIOLOGICAL AGENT (S)

When we first made field surveys of affected fish in Nagasaki Prefecture in November 1996, we found numerous cyst-like organisms in the intestinal tissue and free in

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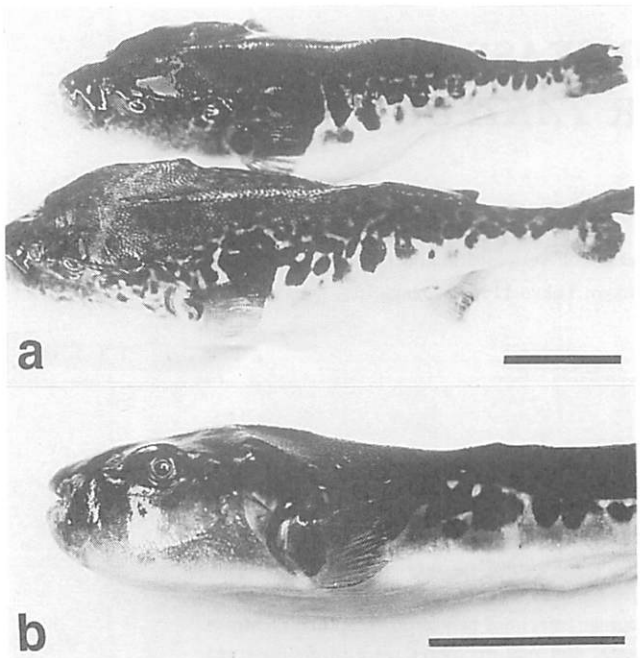
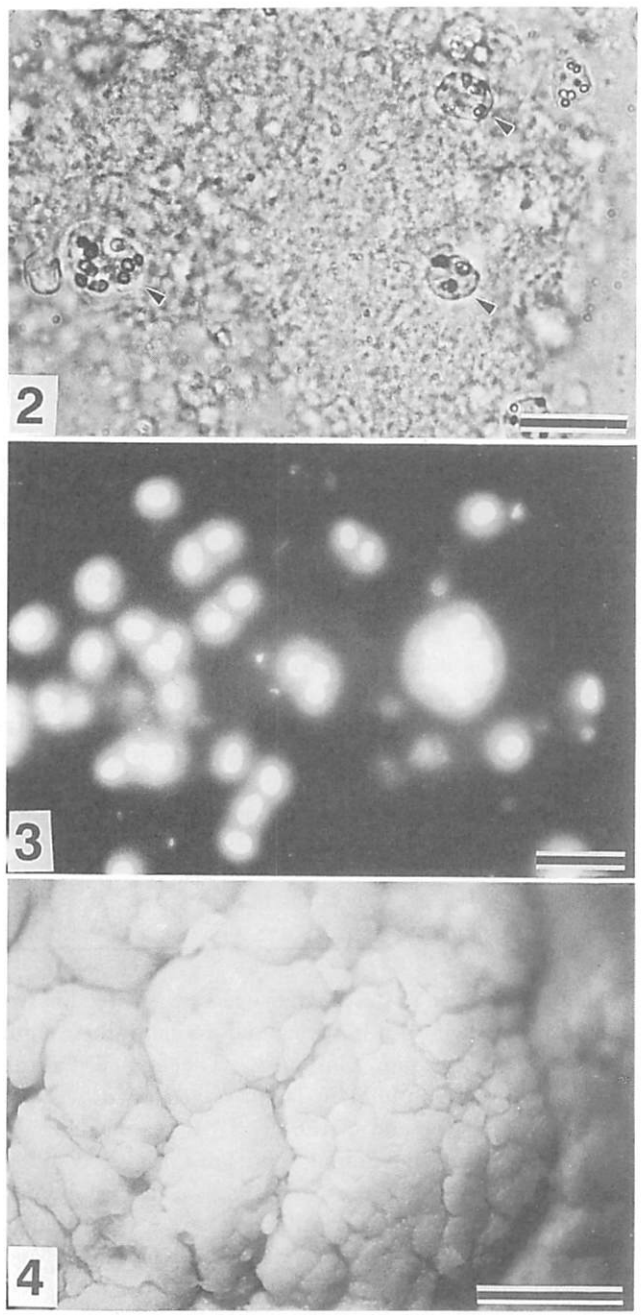


Figure 1. Tiger puffer with clinical signs of severe emaciation. a, obliquely top view; b, side view. Scale bar: 5 cm.

the intestinal lumen of the diseased fish. They were round or subcircular in shape, measuring 5-15 μm in diameter (Figure 2). They contained, in the cytoplasm, spore-like structures with an average size of 3.1 μmm long by 2.3 μmm wide, which were stained by Uvitex 2B, a fluorescent dye which binds specifically to chitin, a component of the microsporidian spore wall (Figure 3) (Yokoyama *et al.*, 1996). Then we thought these cyst-like organisms were host cells of unknown origin infected with microsporeans. Microsporeans contain many fish pathogens (Lom and Dyková, 1992). Thus, we tentatively concluded that the disease was caused by an unidentified microsporean. These studies were presented at the spring meeting of the Japanese Society of Fish Pathology, held at the Tokyo University of Fisheries on March 30, 1997. However, further investigations revealed that the cyst-like organisms were not host cells but myxosporeans infecting the intestine of puffers, characterized by the cell-in-cell structure. This means that the microsporeans are hyperparasites of these myxosporeans.

When we made extensive surveys of emaciated fish in Kumamoto, Nagasaki and Miyazaki Prefectures in 1996-99, we found that, in most cases, they were heavily infected with myxosporeans in the intestine. We found 3 species of myxosporeans in smears of the intestine stained with Diff-Quik or 1% Uvitex 2B and counterstained with 0.5% Evans blue; 2 of them belonged to the genus *Myxidium* and the other *Leptotheca*, all of which had not yet been reported. Tin Tun *et al.* (2000) described *M. fugu* and *L. fugu* as new species, but the other *Myxidium* species was left unidentified because it did not form fully mature spores. It was also revealed that *M. fugu* and *L. fugu* were sometimes hyperparasitized with microsporeans, but that *Myxidium* sp. was not parasitized with them (Tin Tun *et al.*, 2000).

These myxosporean infections with or without hyperparasitic microsporeans had some exceptional fea-



Figures 2-4. Intestine of emaciated tiger puffer. Figure 2: Cyst-like organisms (arrowheads) in the fresh smear of the intestine. Scale bar: 20 μm. Figure 3 : Cyst-like organisms observed with a fluorescence microscope. Apparent microsporean spores are located inside. Uvitex 2B stain. Scale bar: 10 μm. Figure 4: Diseased fish with a gross sign of shortened villi of the intestine. Histological examinations revealed that this gross disease sign was caused by hyperparasitized *Leptotheca fugu*. Scale bar: 1 cm.

tures. First, there was no clear seasonality in the developmental cycle, though myxosporean infections generally showed clear seasonal fluctuations (Lom and Dyková, 1995). From affected tiger puffer, young myxosporean stages were usually detected and mature spores were rarely observed. Secondly, two of the myxosporeans (*Myxidium* sp. and *L. fugu*) are histozoic in the intestine of the host. Similarly, *Myxidium leei* infects the intestinal mucosa of gilt-head sea bream *Sparus aurata* in Israel (Diamant, 1992; Diamant *et al.*, 1994). Intestinal mucosa is an exceptional habitat for myxosporeans.

No gross disease sign was detected in tissues and organs except the intestine, which had often lost its villous structure (Figure 4). Trials to isolate bacterial and viral agents from the intestine or other tissues and organs of diseased fish were not done. The intestine was sometimes infected with *Trichodina*. This is probably a secondary invasion of the ciliate from the external surface of the fish, and there was no clear relationship between the ciliate infection and the disease. No other parasites like *Heterobothrium okamotoi*, a blood feeding monogenean on the gills and branchial cavity wall (Ogawa and Inouye, 1997a, b), appeared to be involved in the disease.

From the above results, it was suspected that at least one, or at most all, of these intestinal parasites (myxosporeans/microsporeans) were involved in the disease condition.

HISTOPATHOLOGY OF THE INTESTINE OF DISEASED FISH

Pathogenicity of these parasites was determined histopathologically (Tin Tun *et al.*, 2001). Tissues and organs of diseased and apparently healthy fish collected from the above localities were fixed in Bouin's solution or 10% formalin, and processed. The sections were cut at the thickness of 5 μm and stained with Giemsa or Uvitex 2B plus hematoxylin and eosin or Azan. The histology slides were examined by light and fluorescence microscopy (UV illumination).

Since diseased fish were often infected with more than one species of myxosporeans in the intestine, fish with a single infection of myxosporean with or without its hyperparasitic microsporean were selected and subjected for histological investigations.

In *M. fugu* infection, parasites attached to the surface of the epithelium, but exhibited negligible pathological changes to its habitat even in heavily infected fish (Figure 5). Only slight host responses were observed. There was no difference in histopathology between fish infected with *M. fugu* and those with microsporean-hyperparasitized *M. fugu* (data not shown).

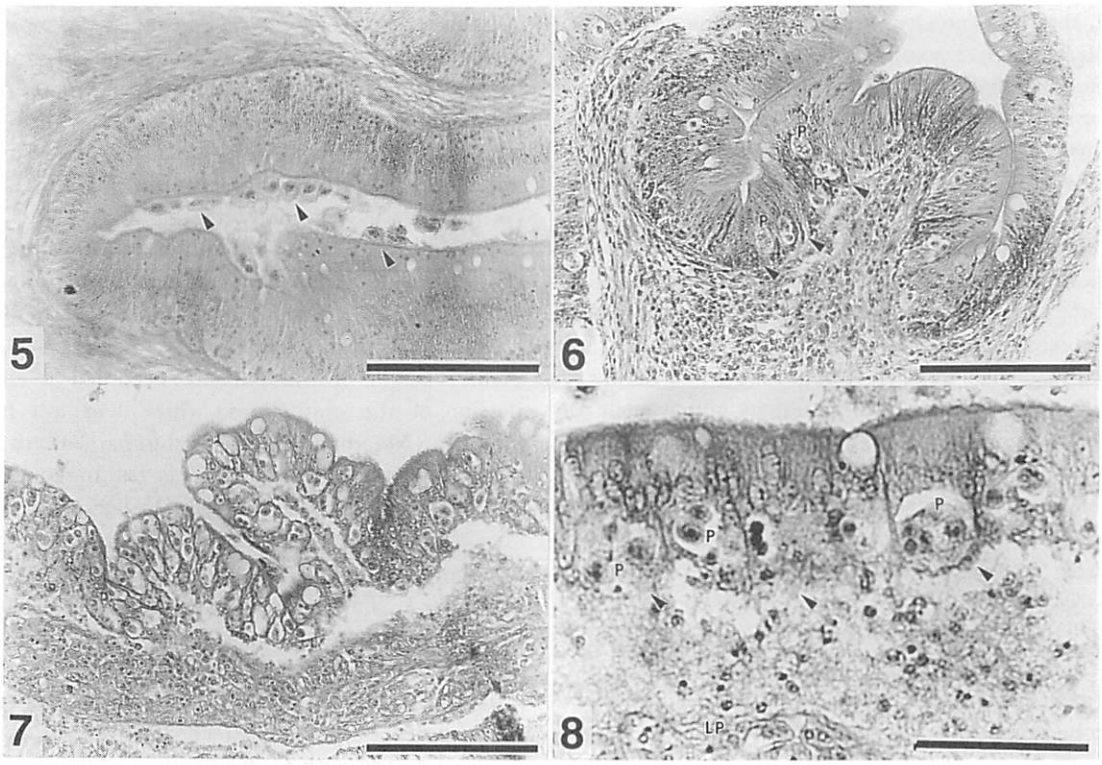
In *Myxidium* sp. infection, host response characterized by patchy infiltration of macrophages into the base of the epithelium was observed (Figure 6). Macrophages attached to the parasite. However, in heavily infected fish, the epithelium became undulated in some places and detached from the lamina propria (Figure 7). Dead macrophages and parasites were accumulated between the epithelium and the lamina propria (Figure 8). Damaged epithelium was eventually degenerated.

In *L. fugu* infection, host responses were similar to those observed in *Myxidium* sp. infection in that macrophages infiltrated into the epithelium, attaching to the parasite (Figure 9). In heavy infections, macrophages and developmental stages of the parasite occupied a considerable portion of the epithelium, which had lost its original integrity. Macrophages attached to parasites to form large aggregates, which were located in the basal region of the epithelium, while, on less frequent occasions, some small ones passed through the basement membrane (Figure 10) and turned into granulomas in the lamina propria and submucosa. The epithelium eventually degenerated, and severe epithelial sloughing was observed locally (Figure 11).

In fish infected with *L. fugu* plus the hyperparasitized microsporean, pathological changes tended to be more severe than fish with *L. fugu* only. Granulomas of various sizes were commonly observed in the lamina propria and submucosa, and degenerated parasites were found inside them (Figure 12). Passages of parasite-macrophage aggregates caused the basement membrane of the epithelium to be discontinuous (Figure 13). Finally, the epithelium degenerated, and sloughed host cells and parasite stages were observed in the lumen. Consequently, intestinal villi became shortened and covered with the decomposed epithelium (Figure 14).

From the above observations, it is evident that *Myxidium* sp. and *L. fugu* with or without the microsporean are highly pathogenic to their host. It is likely that the pathological changes caused by persistent infections with these parasites are responsible for functional disorder in the intestine, such as malabsorption of nutrients and failure in osmoregulation. However, infected fish examined in this study included those which were apparently healthy but heavily infected with *Myxidium* sp. or *L.* sp. (with or without the hyperparasitic microsporean). This is probably because we used naturally infected fish, for which little information was available about the history of infection and the progression of the disease. We have partly succeeded in experimental infections with these parasites by feeding infected tissues to uninfected puffers (T. Yanagida, unpublished data). Experimental data will help to fully understand the role of these myxosporeans in the progression of emaciation in the infected fish.

Similar to the present disease, Branson *et al.* (1999) reported that turbot *Scophthalmus maximus* cultured in Spain showed clinical signs of anorexia, sunken eyes and a prominent bony ridge on the skull caused by unidentified



Figures 5-8. Histology of the intestine of tiger puffer infected with *Myxidium fugu* and *Myxidium* sp. Figure 5: *M. fugu* plasmodia (arrowheads) on the surface of the intestinal epithelium, showing negligible pathological changes. Hematoxylin and eosin stain. Scale bar: 100 μm. Figure 6: Patchy infiltration of macrophages (arrowheads) into the base of the epithelium in the intestine of *Myxidium* sp.-infected fish. P: *Myxidium* sp. plasmodia. Hematoxylin and eosin stain. Scale bar: 100 μm. Figure 7: Undulation and detachment of the epithelium in the intestine of *Myxidium* sp.-infected fish. Hematoxylin and eosin stain. Scale bar: 100 μm. Figure 8: Accumulation of dead macrophages and parasites between the intestinal epithelium and the lamina propria (LP) of *Myxidium* sp.-infected fish. P: *Myxidium* sp. plasmodia. Arrowheads indicate the position of the basement membrane. Hematoxylin and eosin stain. Scale bar: 40 μm.

Myxidium infection in the intestine. Their parasite was morphologically different from *Mxidium* sp. of tiger puffer. Heavy infection induced detachment of the intestinal epithelium and edema and hemorrhages in the submucosa. Although the pathological features in the intestinal submucosa were not obvious in our case, the histological changes in the intestinal epithelium of turbot were quite similar to those described in the *Myxidium* sp. infection of tiger puffer.

ORIGIN OF THE PARASITES

The earliest materials of emaciated tiger puffer were collected from two localities in Kyushu in 1992. Histological examinations revealed that affected fish in Kumamoto Prefecture in August 1992 was infected with the histozoic *Myxidium* sp. and those in Kagoshima Prefecture in November 1992 with *M. fugu* and *Myxidium* sp. Tiger puffer infected with *L. fugu* were collected for the first time by us in Nagasaki Prefecture in November 1996. We are not sure if these parasites are specific to tiger puffer, though they have not been found in any other species of fish. It is not known, either, if they infect not only cultured puffer but also wild ones. With all these results and information, nothing is clear about the origin of these parasites. Our results indicate that all the parasites are already widespread in the Kyushu area. Transportation of infected fish to uncontaminated culture sites, if any, will spread the disease.

In conclusion, the results of the histological examinations show that *M. fugu* and *L. fugu* are pathogenic to host fish. We suggest that they are the etiological agents of the emaciation disease.

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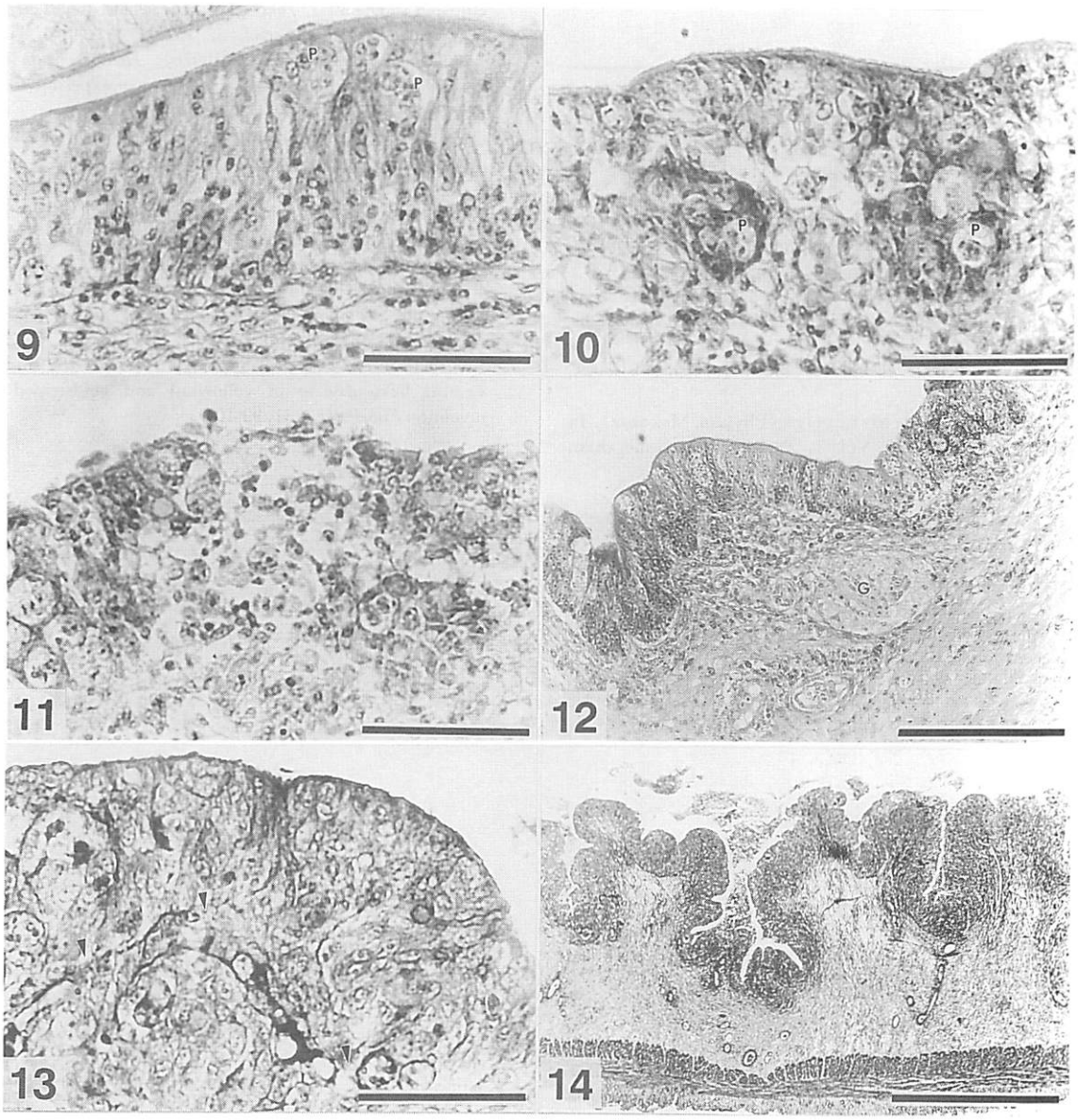

Figures 9-14. Histology of the intestine of tiger puffer infected with *Leptotheca fugu* with or without the hyperparasitic microsporean. Figure 9: Infiltration of macrophages into the intestinal epithelium, attaching to *L. fugu* plasmodia (P). Scale bar: 40 μm. Figure 10: Formation of macrophage-parasite aggregates in the basal region of the epithelium of *L. fugu*-infected fish. Each aggregate contains several *L. fugu* plasmodia (P). Hematoxylin and eosin stain. Scale bar: 40 μm. Figure 11: Degenerating and sloughing intestinal epithelium heavily infected with *L. fugu*. Hematoxylin and eosin stain. Scale bar: 40 μm. Figure 12: Granulomas (G) formed in the lamina propria of the intestine infected with microsporean-hyperparasitized *L. fugu* in the intestine. Hematoxylin and eosin stain. Scale bar: 100 μm. Figure 13: Discontinuous basement membrane (arrowheads) of the epithelium after passages of macrophage-parasite aggregates in tiger puffer infected with microsporean-hyperparasitized *L. fugu* in the intestine. Azan stain. Scale bar: 40 μm. Figure 14: Shortened intestinal villi covered with the decomposed epithelium in tiger puffer infected with microsporean-hyperparasitized *L. fugu* in the intestine. Hematoxylin and eosin stain. Scale bar: 40 μm.

Kumamoto Prefecture, for providing materials of infected tiger puffer.

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INHERITANCE OF RESISTANCE TO *CERATOMYXA SHASTA* IN PROGENY FROM CROSSES BETWEEN HIGH- AND LOW-SUSCEPTIBILITY STRAINS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT

Susceptibility to *Ceratomyxa shasta* among a susceptible and resistant strain of rainbow trout (*Oncorhynchus mykiss*) and their reciprocal F1 progeny was measured as mortality following natural exposure to the parasite. Mortality from *C. shasta* among progeny of the susceptible parental line was 75% following a 3-d exposure, and reached 98% after a 5-d exposure. Mortality from *C. shasta* in all genetic groups with at least one parent of the resistant strain remained below 5% regardless of exposure duration. These data indicate that resistance is a dominant trait for the crosses tested. The identification of a rainbow trout model in which resistance to a pathogen is passed as a dominant trait will be valuable for studies mapping genes for disease resistance and in the identification of the physiological response that dictates the trait.

Key words: *Ceratomyxa shasta*, disease resistance, inheritance of resistance, myxosporea, *Oncorhynchus mykiss*

INTRODUCTION

One of the best documented examples of heritable disease resistance in fish are populations of salmon and trout that have evolved in the presence of the myxozoan parasite *Ceratomyxa shasta*. The geographic range of this parasite is restricted to the Pacific Northwest of the United States and Canada, but within this area it is enzootic in five major river drainages: the Sacramento, Klamath, Rogue, Columbia, and Fraser (Hoffmaster *et al.*, 1988 ; Bartholomew *et al.*, 1989a; Hendrickson *et al.*, 1989). Similar to other myxozoan life cycles described (reviewed by Kent *et al.*, 1994; Uspenkaya, 1995; Yokoyama *et al.*, 1995; El-Mansy *et al.*, 1998 ; Canning *et al.*, 1999 ; Lin *et al.*, 1999; Szekely *et al.*, 1999 ; Ozer and Wooten, 2000), *C. shasta* requires two hosts in which development of alternating spore stages occur. Development of the actinospore stage takes place in a freshwater polychaete worm (*Manayunkia speciosa*) and most species of salmonid can serve as host for the myxospore stage of the parasite (Bartholomew *et al.*, 1997).

Infections by *C. shasta* in susceptible fish are characterized by lethargy, darkening of the body surface, abdominal distension, and hemorrhaging in the area of the vent (Bartholomew *et al.*, 1989b). These signs develop as the parasite invades the intestinal tract, eliciting an inflammatory reaction and causing necrosis of the tissue and eventual death of the host. Variation in the susceptibility of different salmonid species to infection by this parasite and in the development of clinical disease signs was noted in the first descriptions of disease epizootics (Conrad and Decew, 1966; Schafer, 1968; Sanders *et al.*, 1970). Schafer (1968) also reported that "wild" rainbow trout were more resistant to infection by *C. shasta* than the "domesticated" strain. This was the first note of intraspecific variations in susceptibility. The importance of these observations was realized by fishery managers, and numerous species and strains of salmonids were tested for their relative susceptibility to *C. shasta* (reviewed by Bartholomew, 1998). These studies resulted in identification of strains of salmon

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and trout that could survive in enzootic areas because of their low susceptibility to *C. shasta*, and in the development of specific management recommendations for stocking based on the hypothesis that resistance develops as a result of natural selection in enzootic areas (Buchanan *et al.*, 1983).

Identification of differences in susceptibility to *C. shasta* led to investigation on how this trait is inherited (reviewed by Bartholomew, 1998). In each of these studies, F1 progeny produced from reciprocal crosses between high- and low-susceptibility strains of coho salmon (Hemmingsen *et al.*, 1986), steelhead trout (Wade, 1987), and rainbow trout (Ibarra *et al.*, 1992) were intermediate in their susceptibility to infection by *C. shasta* when susceptibility was measured by mortality. Ibarra *et al.* (1994) proposed the hypothesis that genetic control of susceptibility involves multiple loci. However the studies described did not attempt to identify the protective responses involved or the chromosomal regions or genes responsible for resistance. The objective of the study we describe here is to establish a quantitative trait that varies between two strains and can serve as a marker for identifying loci and genes responsible for resistance.

METHODS

Production of Crossbred Strains

Two strains of rainbow trout, a strain with high resistance to *C. shasta* infection (R; Klamath strain) and a strain highly susceptible to the parasite (S; Cape Cod strain), were used as broodstock to produce progeny of the two pure strains (RxR; SxS) and their reciprocal F1s (RxS; SxR). Eggs from eight females of each strain and milt from an equal number of males were collected in late December 1997 and transported on ice from the hatcheries to the Salmon Disease Laboratory (SDL) at Oregon State University, Corvallis, OR. Eggs from each susceptible female were sorted into two groups and fertilized with milt from either a resistant or a susceptible male, creating 32 family groups. Fertilized eggs were incubated in trays (Heath Tecna, Inc.) supplied with 12.5℃ fish pathogen-free water until hatching. At eyed stage, a portion of the eggs from each individual family was transported to the Abernathy Fish Technology Center. After hatching, these families were reared separately in water free of *C. shasta*. The remaining eggs at the SDL were pooled in each of the four family groups. After hatching, progeny were reared at each facility in water free of *C. shasta*.

Infection of Fish with Ceratomyxa shasta

In July 1998, 100 fish from each of the four pooled family groups (RxR ; SxS ; RxS ; SxR) were exposed to the infective stage of *C. shasta* by holding for 3 d in a live cage in the Willamette River, OR (river kilometer 208), where *C. shasta* is enzootic (Zinn *et al.*, 1977). An equal number of unexposed fish from each family served as a control. In September 1998, all remaining unmarked fish (approximately 250 per pooled family group) were exposed for 5 d. Lower river water temperatures at this time permitted a longer exposure without increased mortality from causes unrelated to parasite infection.

To determine the variability in resistance within a genetic group, 25 fish from each of the 32 families reared at the Abernathy facility were marked with a distinctive freeze brand and combined for exposure to *C. shasta*. Exposures were conducted as described and all fish were removed from the river after 3 d.

Following exposure, all fish were transported to the SDL and held in 100-L tanks with 12℃ specific pathogenfree water. Fish were fed a maintenance diet of Oregon Moist Pellet containing 3% oxytetracycline in the form of TM_{100} (Pfizer, Atlanta, GA) to inhibit bacterial pathogens. These treatments are standard procedure and oxytetracycline has no inhibitory effect against *C. shasta* (Udey *et al.*, 1975). Mortalities were monitored daily and dead fish were examined for presence of *C. shasta* spores. Each experiment was terminated at 90 d post-exposure and all surviving fish and controls were killed and examined.

Diagnosis of Ceratomyxa shasta Infection

For each fish, mucosal material was scraped from the posterior end of the intestine using an inoculating loop and a wet mount was prepared. The specimen was examined immediately by bright field microscopy at 250-400 × magnification for 5 min or until *C. shasta* spores were observed (Hendrickson and Bartholomew, 1994). Fish with spores or with obvious sporogonic stages of *C. shasta* were considered positive.

RESULTS

Relative Resistance of Family Groups

Mortality from *C. shasta* began on day 32 post-exposure for fish from the pooled family groups held in the river for 3 d. Incidence of *C. shasta* in each family was as a follows: RxR - 1.7%; RxS - 3.5%; SxR - 0.9%; and SxS - 72.6%. Prior to the time when the first spores were detected, mortality in the RxR, RxS, and SxR families was high (56%, 11%, and 26%, respectively), and no specific etiological agent was diagnosed. Parasite stages were not detected in these fish and the cause of these early deaths was believed to be other than from *C. shasta*. When the data was adjusted to omit deaths that occurred before the

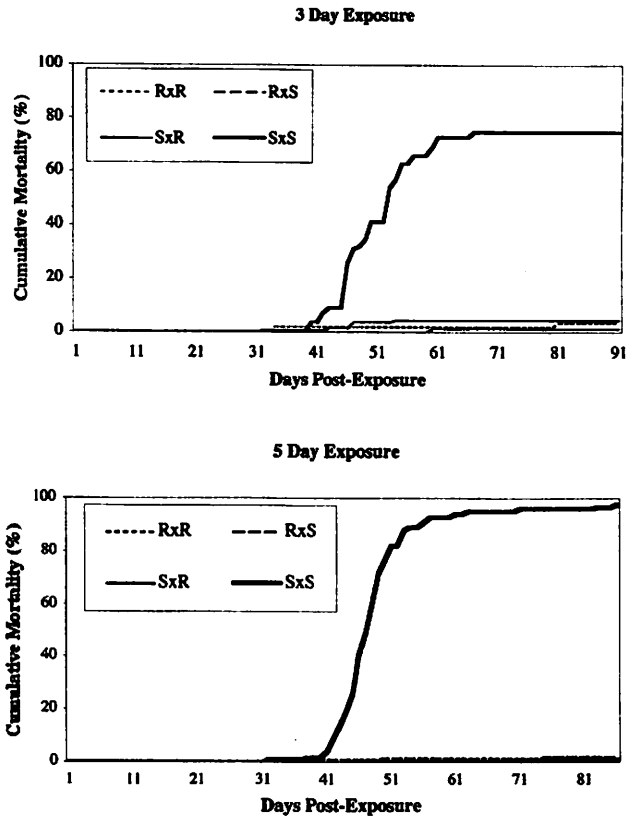

Figure 1. Cumulative mortality from *Ceratomyxa shasta* in progeny of crosses between a resistant (R) and susceptible (S) strain of rainbow trout (*Oncorhynchus mykiss*). The top Figureure graphs mortality following a 3-d exposure to the infective stage and the bottom Figureure is mortality following a 5-d exposure.

infection was patent, prevalence of *C. shasta* was: RxR - 3.8%; RxS - 4.5%; SxR - 1.2% ; and SxS - 75.2 % (Figure 1A). No control fish were diagnosed with *C. shasta*.

Nonspecific mortality following the 5-d exposure was decreased, with the only cases occurring in the RxR group. The first mortality positive for *C. shasta* was on day 24 and the incidence of *C. shasta* in each family, after the data was adjusted to omit the deaths that occurred prior to this time, was as follows: RxR - 2.2%; RxS - 0.4%; SxR - 1.5%; and SxS - 97.7% (Figure 1B).

Variability of Resistance Between Individual Families

Mortality from *C. shasta* began on day 41 post-exposure Incidence of *C. shasta* in all families with at least one parent of the resistant strain (RxR, RxS, SxR) was less than 1% and therefore no variability in resistance between families was discernible. Among the eight SxS families, incidence of *C. shasta* ranged from 87.5% to 100%.

DISCUSSION

The evolution of resistance to *C. shasta* as a result of the strong selective pressure by this parasite has affected the management of fisheries in enzootic regions (Zinn *et al.*, 1977). Exploiting these natural variations in resistance has been proposed as a potential means of controlling the effects of the parasite and the inheritance of resistance has been examined in several studies. In each of the studies reported (Hemmingsen *et al.*, 1986; Wade, 1987; Ibarra *et al.*, 1992), first-generation crosses of high- and low-susceptibility strains of rainbow trout were intermediate to the parental strains for that trait. The implication of these studies for management purposes was that programs for stocking highly susceptibility strains of salmonids in non-enzootic waters were likely to be unsuccessful. An additional concern was that these introductions may adversely affect the fitness of resident strains if interbreeding occurred. Currens *et al.* (1997) presented evidence for this in their study on the susceptibility of wild rainbow trout to genetic introgression and ceratomyxosis as a result of stocking non-indigenous hatchery rainbow trout in the Deschutes River, Oregon USA.

In contrast to these reports, in the present study resistance to *C. shasta* appeared as a dominant trait among progeny from crosses between the resistant Klamath and susceptible Cape Cod rainbow trout strains. The difference between these results and those of the earlier studies could suggest that resistance evolves by different mechanisms in different populations. Resistance to a pathogen is a complex phenomenon which may vary for multiple reasons: physical barriers, destructive effects of mucus or gastric secretions, inactivation by innate mechanisms or targeting by induced mechanisms (Price, 1985; Chevassus and Dorson, 1990). Bartholomew *et al.* (1989b) proposed that in resistant strains penetration or invasion of the infective stage of the parasite was prevented by a barrier at the site of entry. Ibarra *et al.* (1994) supported this hypothesis and based on their results speculated that resistance may also be affected by a second interacting mechanism that involves the ability of the fish to mount an effective immune response against the parasites that succeeded in invading and/or establishing in the host. They suggested that histological evidence of granulomas surrounding the parasite (data not presented) were evidence of this latter mechanism. Histological evaluation of resistant strains in this laboratory (Bartholomew *et al.*, 1989b; present study, unpublished data) has demonstrated the parasite on the surface of the intestinal epithelium, and thus provides support for the barrier mechanism. However, as these populations evolved resistance to *C. shasta* in isolation from each other, the possibility that their primary defenses may vary cannot be discounted.

Duration of exposure and exposure dose are factors that can profoundly influence susceptibility to a pathogen.

Length of exposure has been demonstrated to affect both the incidence of infection by *C. shasta* and the severity of disease, as measured by time to death (Ratliff, 1981; Hemmingsen *et al.*, 1986). When Ibarra *et al.* (1992) measured susceptibility of their rainbow trout crosses by time to death following either a short (7 d) or continuous exposure to the infective stage of the parasite, the first-generation progeny showed little difference from the resistant parental strain. Ibarra *et al.* (1994) concluded that the nature of the genetic causal components differed with time of exposure to *C. shasta*. This hypothesis could not be confirmed in our study because of the poor survival of the *C. shasta*-resistant strain during and following the natural exposure. Although the cause of death was not determined, most mortalities occurred prior to the period when infection by *C. shasta* becomes patent. It has been reported that enhanced resistance to a disease may not be correlated with improvements of other fitness traits (Price, 1985) and this may contribute to these results. All fish were hatched and reared under identical conditions, and differences between the susceptible and resistant strains were physically apparent throughout the study. It is possible that the Klamath strain is uniquely suited to its natural habitat and therefore less suited to perform under the conditions of this study than the domesticated Cape Cod strain.

One factor that was not examined in previous studies was the variability in susceptibility between family groups. In this study, eight families for each of the four crosses (RxR, RxS, SxR, SxS) were exposed to *C. shasta*. Mortality among progeny with at least one parent from the resistant strain was less than 1%, and mortality in progeny from the crosses between susceptible parents was between 87-100%. Because the infectious dose cannot be determined in natural exposures, it is not possible to determine if these small differences are a result of individual variation or of differences in exposure dose or duration. However, the lack of significant differences among the reciprocal F1 progeny in this study lends support to the hypothesis that sex effects are not involved (Hemmingsen *et al.*, 1986; Ibarra *et al.*, 1992).

The identification of a *C. shasta*-rainbow trout model in which resistance is passed as a dominant trait will be useful for future studies mapping the genes responsible for this trait. Clonal lines of the Klamath strain have been produced (collaboration with Dr. Gary Thorgaard, Washington State University) and application of genetic linkage mapping tools (Young *et al.*, 1998) in combination with detailed immunological investigations should result in identification of resistance mechanisms and the genes that control them. Identification of these genes will be valuable not only in breeding programs, but will be useful for selecting markers for disease-rasistant stocks, allowing better management of valued wild populations.

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ANEMIA OF JAPANESE FLOUNDER CAUSED BY THE MONOGENEAN *NEOHETEROBOTHRIUM HIRAME*

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ABSTRACT

Anemia with unknown etiology has been prevailing in both wild and cultured flounder in Japan since the mid-1990s. Although the cause of the anemia has been a matter for argument over the years, several research groups recently offered convincing data that the monogenean *Neoheterobothrium hirame* is the causative agent of the anemia. Moreover, information on the anemia and the parasite has been accumulated. In this paper, the studies and information are summarized and reviewed from the viewpoints of pathology, etiology, diagnosis and epidemiology.

Key words: monogenea, *Neoheterobothrium hirame*, Japanese flounder, *Paralichthys olivaceus*, anemia

INTRODUCION

Recently, anemia with an unknown etiology suddenly appeared and has been prevailing in both wild and cultured Japanese flounder *Paralichthys olivaceus* in Japan. Since the Japanese flounder is one of the most important fish species in Japan, the anemia has been capturing the attention of fishermen, fish culturists, researchers and administration officers. Although there have been arguments on the causative agent of the anemia, it was recently clarified, based on studies by several researching groups, that *Neoheterobothrium hirame*, a monogenean parasite, is the cause of the anemia. Moreover, information on the anemia and the parasite has been accumulated. The purpose of this paper is to summarize and review the studies and information.

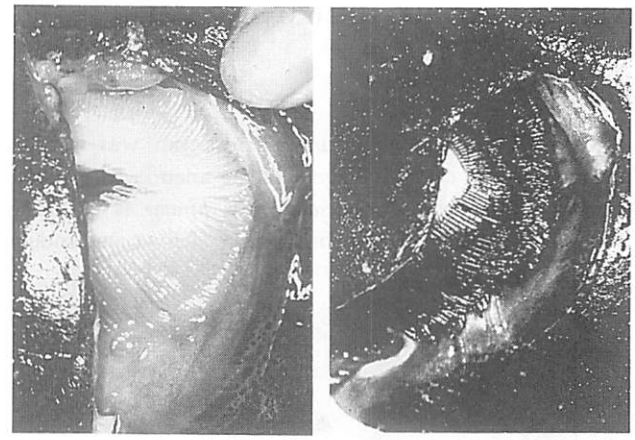

Figure 1. The gills of an anemic flounder (left) and a healthy flounder (right).

CHARACTERISTICS OF THE ANEMIA

Gross observation

Anemic flounder lose the reddish color in the gills. Heavily anemic flounder having yellow gills are still alive and often captured from the sea (Figure 1) . The lower side of the body of heavily anemic flounder is pale colored, while that of healthy flounder is yellowish white. The viscera such as the liver, spleen and kidney are decolorized.

Hematology

The hemoglobin concentrations in the peripheral blood of anemic flounder often reach as low as below 1 g/dL, while those of healthy flounder range from 3 to 9 g/dL. The hematocrit values of anemic flounder are mostly below 15%, while those of healthy anemic flounder mostly

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range from 15 to 35%. However, the erythrocytes in heavily anemic flounder are not packed well in hematocrit capillary tubes and form spaces in the tube walls, which make the precise determination of hematocrit values difficult. In the peripheral blood of anemic flounder, immature erythrocytes are frequently observed (Figure 2). The ratios of immature erythrocytes to total erythrocytes occasionally reach as high as over 75%. Abnormal immature and mature erythrocytes having vacuolated or weakly stained cytoplasm are frequently observed. Deformed erythrocytes having processes at one or both ends are occasionally observed as well.

Histology

Marked histological lesions are absent in anemic flounder. Although Miwa and Inouye (1999) reported the presence of pyknotic cells in the hematopoietic tissues in the spleen and kidney of anemic flounder, they are scarce and dispersed, seemingly not involved in the anemia. Neither marked hemosiderin deposition nor necrotic lesions, which are often observed in viral infections, are present in the hematopoietic tissues.

NEOHETEROBOTHRIUM HIRAME

Etiology

Although a virus or virus-like organism was initially proposed as the causative agent of the anemia, the blood-feeding monogenean *Neoheterobothrium hirame* is currently thought to be the cause of the anemia. Miwa and Inouye (1999) reported the presence of virus or virus-like particles in the hematopoietic tissues of anemic flounder by electron microscopy, suggesting that a virus may be the cause of the anemia. However, such a virus or virus-like organism was observed also in healthy flounder (personal communication; Nakayasu, C., National Research Institute of Aquaculture, Mie, Japan). Moreover, several attempts to produce anemic fish by injections of the tissue homogenate or blood taken from affected fish failed (personal communication; Miura, H., Hokkaido Prefectural Fish Hatchery, Hokkaido, Japan). On the other hand, anemic flounder were infected with *N. hirame* at very high prevalences (over 70%) on the buccal cavity wall of both wild and cultured populations. Yoshinaga *et al.* (2001) attempted experimental challenges with *N. hirame* to Japanese flounder and successfully produced anemic flounder that had hematological characteristics very similar to those in anemic flounder in the wild or cultured populations. Moreover, hematological characteristics of the anemia were produced by repeatedly taking blood from flounder (personal communication; Nakayasu, C.). These data strongly suggest that the anemia is caused by the blood-feeding activity of the parasite. The absence of histological lesions indicating viral infection supports the suggestion.

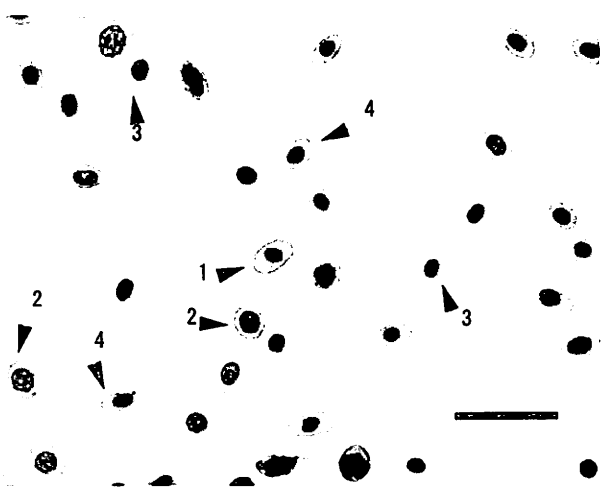

Figure 2. The blood smear of an anemic flounder. 1, normal erythrocyte. 2, abnormal immature erythrocytes having vacuolated or weakly stained cytoplasm. 3, abnormal mature erythrocytes having weakly stained cytoplasm. 4, deformed erythrocytes. The bar is 20 μm.

Anemic flounder with no *N. hirame* on the buccal cavity wall are often found in wild populations in winter (personal communication; Higuchi, M., Niigata Prefectural Fisheries and Marine Research Institute, Niigata, Japan). This observation is inconsistent with the suggested cause of the anemia in the Japanese flounder, offering another suggestion that a causative agent other than *H. hirame* may also be involved in the anemia. However, most of the wild anemic flounder without the parasite have nodules containing worm body debris beneath the buccal cavity wall as the trace of infection (Yoshinaga, 2000a) (personal communication; Kumagai, A., National Research Institute of Aquaculture, Mie, Japan). The detachment of worms may be an explanation for the anemic flounder without worms in wild populations.

Neoheterobothrium hirame was discovered from Japanese flounder in 1993 as a new species (Ogawa, 1999). The parasite has been recorded only from Japanese flounder so far. The hatched larvae, or oncomiracidia, of the parasite settle on the gill filaments of flounder and grow there feeding blood. The worms migrate to the wall of the upper jaw or the wall between the pharyngeal teeth in the buccal cavity, where they insert the haptor into the muscular layer beneath the wall and feed blood from the gill (Figure 3). The matured worms on the buccal cavity wall are 10-20 mm long, grayish brown in color, and easily observed. However, when examining heavily anemic flounder, careful observation is needed, because the worms on heavily anemic fish are whitish in color, making the examination difficult.

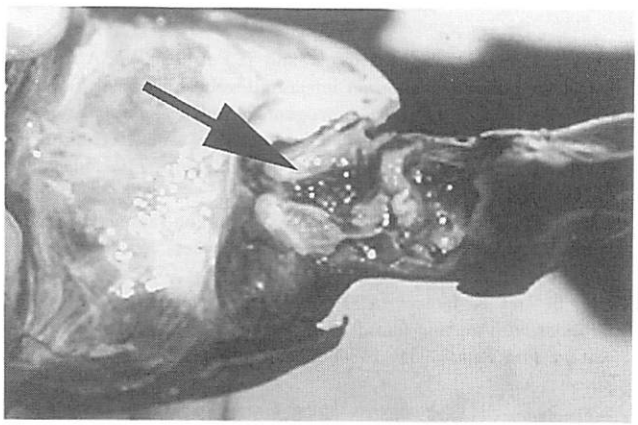

Figure 3. The cluster of *Neoheterobothrium hirame* (arrow) between the pharyngeal teeth of Japanese flounder.

Diagnosis

The confirmation of the presence of *N. hirame* is obviously the first step for the diagnosis. However, anemia caused by viral or bacterial infections is commonly observed especially in culture facilities. Therefore, routine examination for viral and bacterial infection is needed for the confirmative diagnosis of cultured anemic flounder. When viral or bacterial examination is difficult, such as in captured wild flounder, histological or hematological examination may be the alternative.

Distribution

The Fish Pathology Division, National Research Institute of Aquaculture (NRIA), sent inquiries to local fisheries experimental stations in the spring of 1998 to investigate the occurrence of anemic flounder and *Neoheterobothrium hirame*. According to the results of the investigation, wild anemic flounder appeared for the first time in the Sea of Japan in 1993. The parasite was first recorded from a coastal water in the Sea of Japan in 1993. Until 1998 or the time of the investigation, anemic founder and *N. hirame* had been widely distributed along the coast of Honshu on the Sea of Japan. However, on the coast of the Pacific, they had been dispersedly found only in several areas until 1998. Since then, wild anemic flounder infected with the parasite have been reported from many areas on the coasts along the East China Sea and the Pacific as well. Nowadays, anemic flounder infected with *N. hirame* are frequently reported throughout Japan, except Hokkaido, the Okinawa islands and the Amami islands. The investigation by NRIA prompted the researchers at the local fisheries experimental stations to examine anemic flounder and *N. hirame*, probably resulting in the frequent reports on anemic flounder. However, it seems that anemic flounder infected with *N. hirame* appeared initially in the Sea of Japan and expanded the distribution to the Pacific coast. In culture facilities on the coast where *N. hirame* exists, anemic flounder infected with the parasite have been commonly observed.

Prevention and control

In Japan, Japanese flounder are cultured mostly in land-based tanks using seawater. The eggs or hatched larvae are introduced in the culturing facilities along with the seawater, and thus infect cultured flounder. Although elimination of the worm eggs and larvae from the water would be the most effective method for the prevention of *N. hirame* infection, conventional sand filtration does not work well to eliminate the eggs and larvae from the water; they seemingly pass through sand filters (personal communication; Yamamoto, E., Tottori Prefectural Fisheries Station, Tottori, Japan). Because the eggs of *N. hirame* sink on the bottom of tanks and are easily removed from tanks with wastewater, placing water outlets on the bottom of tanks and increasing water exchange rates are effective methods in decreasing the chance of propagation of *N. hirame* in tanks, resulting in the decrease of damage by the parasite (personal communication; Yamamoto, E.). Immature worms on the gills can be removed by bathing infected fish in seawater supplemented with 3% NaCl (Yoshinaga *et al.*, 2000b). However, no effective treatment has been developed to kill mature worms on the buccal cavity wall.

In Japan, more than 20 million juvenile flounder are released into natural waters for the stock enhancement of flounder. For the production of the released juveniles, wild flounder captured from natural waters are used as their parents to preserve the genetic diversity. However, wild flounder are often infected with *N. hirame* and become anemic when reared in tanks. To prevent anemia and *N. hirame* infection in the parental flounder, the mechanical removal of mature worms by forceps and the bathing in NaCl-supplemented seawater are often employed.

Impact on wild flounder

The annual catches of Japanese flounder increased in Japan year by year from 1990 to 1997. In 1997, the catch reached 8400 metric tons. The increase is believed to be the results of the recent mass release of flounder juveniles and of fish size regulations in catching. However, the catches have clearly decreased since 1998, despite the continuation of the release and size regulation; the catch decreased to 7200 metric tons in 1999. Especially in the western part of the Sea of Japan, the annual catches began to decrease in 1996, several years later than the appearance of anemic flounder; the catches decreased from 930 to 380 metric tons between 1995 and 1999. In most areas in Japan, the catches similarly began to decrease several years later

than the appearance of anemic flounder. On the other hand, in an area in the Sea of Japan, the prevalence of infection with the parasite among wild flounder populations once reached nearly 90 % (Anshary *et al.*, 2001). It is therefore a growing concern that *N. hirame* infection may be involved in the decreased catches of flounder, and further studies are needed to clarify the cause of the recent decrease in flounder catches.

Origin

Neoheterobothrium hirame was discovered in 1993 as a new species. Considering that Japanese flounder is one of the most studied fishes in Japan, it is unlikely that the parasite had been present long before the discovery. Generally, populations of fish parasites largely fluctuate especially in closed waters depending on environmental changes. However the sudden appearance of *N. hirame* cannot be explained by any environmental changes, because it occurred widely in open waters from northern Honshu to southern Kyushu. The most probable explanation for the appearance of the parasite is that this parasite was recently introduced into adjacent waters around Japan from another part of the world, and further studies are needed to clarify the origin of the parasite.

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A REVIEW OF POTENTIAL DISEASES OF BLUE CRABS

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ABSTRACT

Blue crabs *Callinectes sapidus* are an important economic and recreational resource on the Atlantic and Gulf coasts of the USA. Blue crabs are caught in the wild and sold either as hard or softshell crabs. Hard crabs are steamed and eaten by picking the tender white meat from the shell. Softshell crabs, a seafood delicacy, are caught as pre-molts and held in captivity until they shed their old exoskeleton, exposing a soft new shell. Disease can cause mortalities, decrease fecundity, or reduce demand in both wild populations and in crabs contained in holding facilities. Crabs held in captivity are particularly subject to viral and bacterial infections. Viruses which cause mortality include a reo-like virus, a bi-facies virus, and a picorna-like virus. Systemic bacterial infections can be caused by numerous species including *Vibrio parahaemolyticus*. Some of the most harmful pathogens infecting wild populations of blue crabs include microsporidian species; an amoeba *Paramoeba perniciosa*; a dinoflagellate *Hematodinium sp.*; and a ciliate *Mesanophrys chesapeakensis*. Other pathogens decrease fecundity, including the fungus *Lagenidium callinectes*, and the parasitic barnacle *Loxothylacus texanus*. Finally, other diseases reduce marketability of infected crabs. Chitinoclastic bacteria including species of *Vibrio* and *Pseudomonas* produce unattractive necrotic lesions on the exoskeleton of crabs; and a trematode *Microphallus basodactylophallus*, hyperparasitized by the haplosporidian *Urosporidium crescens*, produces undesirable black pigmented spots in the musculature of cooked crabs.

Key words: *Callinectes sapidus*, blue crabs, diseases, mortalities

INTRODUCTION

The blue crab *Callinectes sapidus* is the most valuable commercial and recreational species of crab along the Atlantic and Gulf coasts of the USA. Although both hard-shell and softshell stages of blue crabs are consistently an important fishery resource, the fishery has experienced major fluctuations in landings. Outbreaks of disease combined with environmental factors have been blamed for some variations in crab production. Diseases cause mortalities in both wild populations and in crabs contained in holding facilities (Sprague, 1965; Overstreet, 1977; Johnson, 1983). Diseases affect the blue crab industry by causing mortalities, decreasing fecundity, or decreasing demand. Many microbial diseases have been reported in blue crabs, including those of viral, bacterial, fungal, and protozoan etiologies (Table 1).

VIRAL DISEASES

Viruses from marine invertebrates were unknown before 1966 (Vago, 1966). Today there are 7, possibly 8, viruses reported to infect blue crabs, although not all are pathogenic or cause mortalities. Three viruses that infect blue crabs that are considered lethal include reo-like virus (RLV), bi-facies virus (BFV), and picorna-like Chesapeake Bay virus (CBV). The RLV acts synergistically with the rhabdo-like virus (RhVA) and is responsible for hemocyte (Figure 1) and nerve tissue damage, often resulting in death. This is especially true in premolt crabs about to go through ecdysis (Johnson, 1985). The BFV causes nuclear hypertrophy and lysis of hemocytes (Figure 2) while eventual death is due to hemocyte dysfunction (Johnson, 1978). The CBV causes extensive destruction to gill and bladder epithelium and neurosecretory cells (Figure 3). This virus can cause blindness and death (Johnson, 1978). Viral infections may become patent when crabs have been exposed to stress factors such as captivity, crowding, or a degraded environment (Couch, 1974b; Johnson, 1977a, 1978, 1984, 1985; Yudin and Clark, 1979; Messick and Kennedy, 1990; Messick and Sindermann, 1992).

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Table 1. Disease agents that infect blue crabs.

Agent	Description
Viruses	
RLV + RhVA	Reo-like virus + rhabdo-like virus
CBV	Chesapeake Bay virus; a picorna-like virus
BFV	Bi-facies virus, formerly herpes-like virus (HLV)
Bacteria	
Chitinoclastic	*Vibrio* and *Pseudomonas*
Systemic	*Vibrio parahaemolyticus, Pseudomonas, Acinetobacter, Bacillus, Flavobacterium*, and coliforms including *Escherichia coli*
Fungi	
Lagenidium callinectes	Phycomycetous
Protozoans	
Ameson michaelis	Microspora
Paramoeba perniciosa	Sarcomastigophora
Hematodinium sp.	Sarcomastigophora
Mesanophrys chesapeakensis	Ciliophora
Helminths	
Microphallus spp.	Trematode metacercariae hyperparasitized by *Urosporidium crescens* (haplosporidian)
Crustaceans	
Loxothylacus texanus	Sacculinid barnacle, rhizocephalan

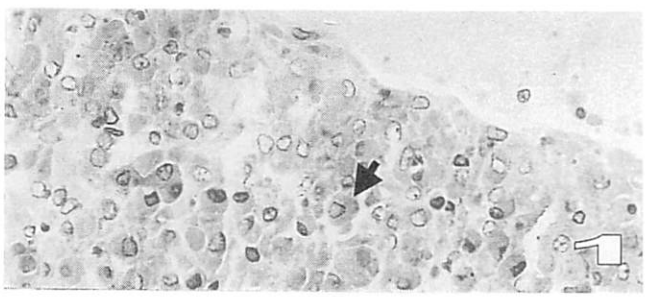

Figure 1. Reo-like-Rhabdo-like virus (RLV-RhVA) infection in hemopoietic tissue of intermolt crab. Note increased cytoplasmic volume (arrow).

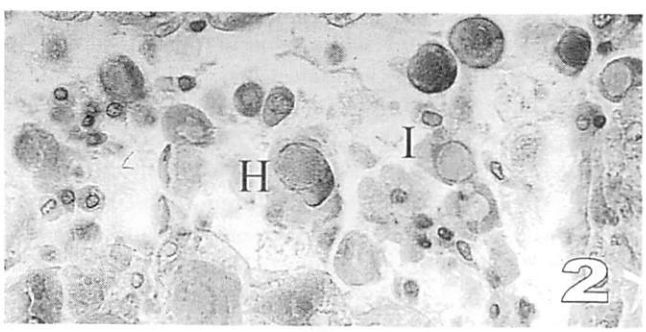

Figure 2. Bi-facies virus (BFV) infecting hemocytes. Note nuclear hypertrophy (H) and cytoplasmic inclusions (I).

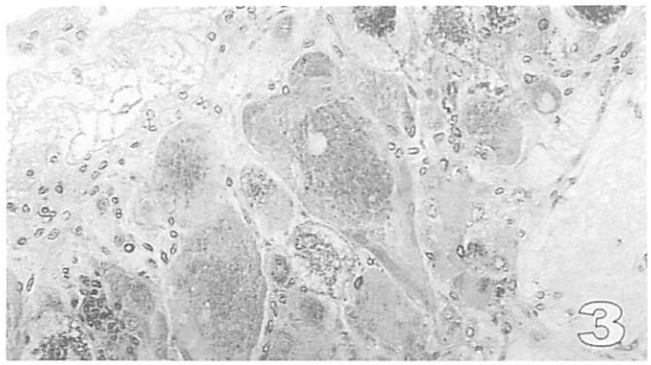

Figure 3. Chesapeake Bay virus (CBV) infecting thoracic ganglion of central nervous system. Note cell enlargement and destruction (arrow).

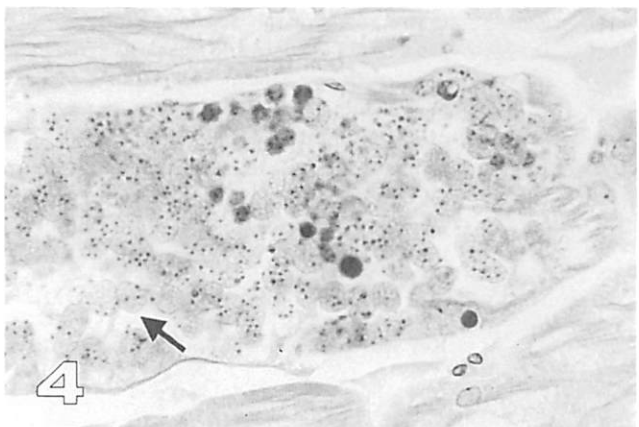

Figure 4. Microsporidian infection in blue crab skeletal muscle. Note bundles of spores (arrow) and individual spores within bundles.

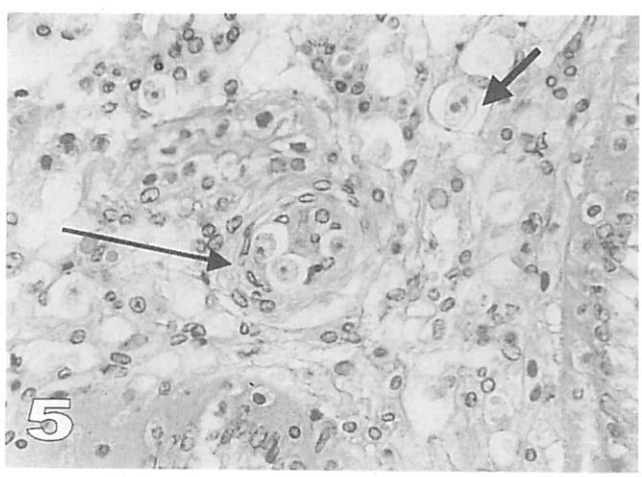

Figure 5. *Paramoeba perniciosa* infection in blue crab tissue. Note halo surrounding individual amoebae (short arrow). Host response includes encapsulation (long arrow).

BACTERIAL DISEASES

Shell disease

Shell disease is one of the most common and widespread bacterial diseases of crustaceans since chitinoclastic bacteria are ubiquitous in marine environments (Benton, 1935; Zobell and Rittenberg, 1938; Hock, 1940, 1941; Lear, 1963; Hood and Meyers, 1974). Shell disease syndrome is characterized by deep necrotic, blackened lesions on various portions of the crab's exoskeleton and is caused by chitinoclastic bacteria and other chitin-destroying microorganisms. The disease syndrome decreases the market value of lightly or moderately infected crabs, or in severe cases, makes them unmarketable. Infected crabs often succumb to secondary infections by parasites which invade tissues through the damaged exoskeleton and may cause mortalities. Deteriorated water quality (Engel and Noga, 1989; McKenna *et al.*, 1990), or crowded conditions in shedding or holding tanks, can cause unusually high incidence of shell disease (Sindermann, 1989). Most chitinoclastic bacteria isolated from lesions in blue crabs belong to several genera including *Vibrio* and *Pseudomonas* (Cook and Lofton, 1973; Johnson, 1983).

Systemic bacterial infections

Many blue crab mortalities are attributed to systemic bacterial infections, especially when the animals are subjected to crowded, confined conditions. Hemolymph from infected crabs has reduced clotting and diminution of hemocyte numbers. Hemocyte aggregations develop in hemal spaces; as infections progress, hemocytic nodules form in various organs (Johnson, 1976). An average of 28% of crabs dredged from Chesapeake Bay, USA had hemocyte aggregations or infiltration and 11% had nodules or encapsulations which were initiated by foreign material such as bacteria (Messick, 1998). Bacteria isolated from the hemolymph of moribund and apparently healthy crabs include *Vibrio parahaemolyticus* (Davis and Sizemore, 1982; Sizemore and Davis, 1985), *Pseudomonas, Acinetobacter, Bacillus, Flavobacterium*, and a heterogenous group of coliforms including *Escherichia coli* (Colwell *et al.*, 1975; Sizemore *et al.*, 1975; Welsh and Sizemore, 1985).

FUNGAL DISEASES

Marked annual fluctuations in commercial catches of blue crabs in Chesapeake Bay, USA have been associated with a parasitic fungus *Lagenidium callinectes* (Couch, 1942) infecting egg masses. Infected eggs are below their normal size and fail to hatch, or abnormal prezoea are produced which rarely survive (Sandoz *et al.*, 1944). Infected eggs have an atypical brown or gray color, depending on the maturity of the eggs. Infection spreads rapidly over the egg mass periphery; penetration into the sponge is slow and shallow. *Lagenidium* can also infect larvae of blue crabs, causing extensive deformities and mortalities.

An unusual strandlike microorganism reported in blue crabs is found in the lumen and attached to hepatopancreas tubules. In tissue sections, the organism appears as a filamentous, nonseptate mass attached by a basal holdfast to hepatopancreas epithelial cells. Infections are focal with only a few tubules exhibiting lesions. An average of 4% (n = 25/657) and up to 16% (n = 11/72) of crabs sampled in autumn in Chesapeake Bay, USA were infected with this unidentified organism (Messick, 1998). It is unknown what effect this organism has on crab health.

PROTOZOAN DISEASES

Many protozoans are known to cause disease in blue crabs, some in epizootic proportions, resulting in mass mortalities, but often enzootics occur, killing only a few. Diseases caused by protozoans often become patent when crabs are held in artificial environments, such as peeler shedding tanks or holding facilities for market. Some of the most pathogenic protozoans found infecting blue crabs are microsporidians (Sprague, 1950, 1970), including *Ameson, Nosema*, and *Pleistophora*; two members of the Sarcomastigophora, *Paramoeba* (Sprague and Beckett, 1966, 1968) and *Hematodinium* (Newman and Johnson, 1975; Newman, 1977; Couch, 1983); and a histophagous ciliate *Mesanophrys chesapeakensis* (Messick and Small, 1996).

Ameson michaelis, A. sapidus, and *Pleistophora cargoi* are 3 microsporidian species which infect blue crabs. Crabs heavily infected with microsporidians are sluggish and have an abnormal appearance; infections can be identified by an opaque white to gray ventral sternum (Sprague, 1950, 1970; Overstreet, 1977). Microsporidian infections cause muscle lysis and replacement of muscle tissue with masses or bundles of spores (Figure 4) (Overstreet, 1978). Infected animals die rapidly if stressed (Overstreet and Whatley, 1975). Microsporidians are potentially very pathogenic when considering the loss of economic potential of reared crabs for the softshell industry. Microsporidians were found in up to 6% (6/101) of crabs held in experimental shedding systems (Messick and Kennedy, 1990), and in up to 10% (22/224) of wild-caught dredged crabs (Messick, 1998). This parasite is easily transmitted from sick and dying infected crabs to healthy crabs. One measure of control is to encourage fishermen to dispose of sick crabs on land rather than throwing them back into the sea. Some measures have been taken to find chemical deterrents to help reduce the spread of the parasite under controlled conditions of captivity (Overstreet and Whatley, 1975).

"Gray crab disease," caused by *Paramoeba perniciosa*

(Sprague and Beckett, 1966, 1968), results in mortalities of wild populations as well as those in shedding systems. Diseased crabs are sluggish and often have a gray ventral surface; hence, the term"gray crab disease." Infected crabs have reduced hemolymph clotting and often die after being stressed by handling (Sawyer, 1969; Sprague *et al.*, 1969; Johnson, 1977b; Couch, 1983). The major diagnostic characteristic of *Paramoeba* is the presence of a secondary body in the cytoplasm. In heavy infections, amoebae replace hemocytes (Johnson, 1977b; Couch, 1983). Host defense to the parasite includes phagocytosis by hyaline hemocytes and hemocytic encapsulation (Figure 5). The cause of death in *Paramoeba* infections is unknown; it may be that loss of vital tissue leads to inanition and organ dysfunction. Epizootics may be major factors in annual winter and late spring mortalities of blue crabs and the parasite may cause chronic low level mortalities in late spring of non-epizootic years (Newman and Ward, 1973; Couch, 1983). Infections and mortalities are most commonly reported from high salinity coastal bays. *Paramoeba* also infects rock crabs *Cancer irroratus*, lobsters *Homarus americanus* (Sawyer and MacLean, 1978), and green crabs *Carcinus maenas* (Campbell, 1984).

Another member of the protozoan phylum Sarcomastigophora recognized as causing disease in blue crabs is the parasitic dinoflagellate *Hematodinium* sp. *Hematodinium* infections can be fatal, but infected crabs exhibit no external signs except lethargy and opaqueness. Hemolymph from heavily infected animals is milky, due to the presence of many dinoflagellates; in some instances, tissues may have a pink opalescent color. The parasite is found primarily in the hemolymph, but is also seen in and between muscle fibrils, gonad, hepatopancreas, and other tissues. The parasite is characterized by apparent continuous mitotic activity in the nucleus and dinokaryon-type nuclei arranged in V-shapes (Figure 6). Heavily infected crabs have reduced hemocyte numbers, with apparent replacement by *Hematodinium* sp. (Figure 6) (Messick, 1994). The disease has been reported in blue crabs from salinities only above 11ppt . Prevalence is highest in crabs collected from waters with salinities of 26 to 30ppt (Messick and Shields, 2000). Reported geographic distribution in blue crabs includes the Atlantic and Gulf coasts of USA (Newman and Johnson, 1975; Newman, 1977; Couch, 1983; Messick, 1994). Prevalence follows a seasonal pattern with a sharp peak in late autumn (Messick, 1994; Messick and Shields, 2000). A host characteristic which influences prevalence is size; infections are significantly more prevalent in crabs measuring less than 30 mm carapace width. Crab sex and molt stage did not influence prevalence. Naturally and experimentally infected crabs die when held over 35 and 55 d in captivity, with a mean time to death of approximately 13 and 42 d, respectively (Messick and Shields, 2000). In the laboratory, experimentally infected crabs suffer mortalities as high as 86% (34/40) due to the disease; this is a rate 7 to 8 times higher than uninfected control animals (Shields and Squyars, 2000). *Hematodinium* spp. have been detected in numerous other crustaceans including *Carcinus maenas*, *Portunus depurator* (Chatton and Poisson, 1930), *Nephrops norvegicus*, and *Necora puber* (Shields, 1994) from Europe. The rock crab *Cancer irroratus*, the Jonah crab *Cancer borealis*, and the lady crab *Ovalipes ocellatus* from the Middle Atlantic Bight of USA's east coast (MacLean and Ruddell, 1978) have been found with infections, and *Chionoecetes bairdi* from Alaska (Shields 1994) are also infected. In Australia, *Trapezia aerolata*, *Scylla serrata*, and *Portunus pelagicus* have been found with *Hematodinium* sp. infections (Shields 1994). Considering its wide distribution and high pathogenicity, *Hematodinium* spp. represent a significant threat to crustacean species.

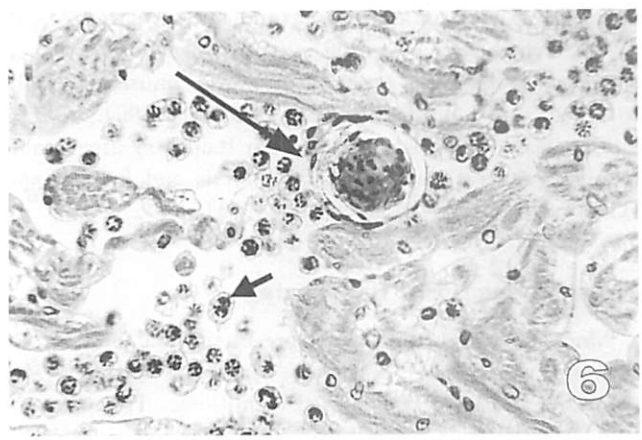

Figure 6. *Hematodinium* sp. infection within hemal sinus of cardiac muscle. Note the numerous uninucleate trophonts (short arrow) and encapsulation by hemocytes, an apparent host response (long arrow).

Figure 7. Blue crab gills with "pepper spots" which are metacercariae of a digenean trematode hyperparasitized by haplosporidian *Urosporidium crescens* (arrow).

Histophagous ciliates from wild and captive crustaceans have been reported from various locations since 1888 (Cattaneo, 1888). Most reports of ciliates in crustacean hemolymph involve captive animals (Cattaneo, 1888; Bang *et al.*, 1972; Aiken *et al.*, 1973; Grolière and Leglise, 1977; Armstrong *et al.*, 1981; Sparks *et al.*, 1982) but infections are found sporadically in wild animals. Infections by the histophagous ciliate *Mesanophrys chesapeakensis* are found infrequently in wild and captive blue crabs (Messick and Small, 1996). Pathology associated with infections include hemocyte aggregations in various tissues and nodule formation. *M. chesapeakensis* likely feeds on the hemolymph, hemocytes, and tissues of infected crabs, causing stress to the host by removing available nutrients from the hemolymph (Messick and Small, 1996).

HELMINTHS

Metacercariae of digeneans *Microphallus basodactylophallus* and *Megalophallus* sp. often parasitize the musculature of blue crabs. These larval flukes are not easily observed unless hyperparasitized by the haplosporidian *Urosporidium crescens*. When hyperparasitized, the metacercariae become enlarged (410-654 μm), are darkly pigmented, and rupture easily; whereas, uninfected or lightly infected metacercariae are smaller (189-269 μm), white to cream color, and can withstand some mechanical pressure (DeTurk, 1940; Couch, 1974a; Overstreet, 1978; Millikin and Williams, 1984). The presence of metacercariae or "pepper spots" in cooked crabmeat is objectionable to consumers and can decrease the desirability and market value of infected crabs (Figure 7).

PARASITIC BARNACLES

A truly parasitic sacculinid barnacle infecting blue crabs is the rhizocephalan *Loxothylacus texanus*. The interna or "root system" of the barnacle penetrates the soft abdominal joint of the crab and is extended into internal tissues when the crab molts. The externa of the barnacle, consisting of a brood pouch for gonads and larvae, protrudes from under the crab's abdomen, appearing as a bulge that can be seen grossly. The barnacle causes a restriction in growth; infected individuals are only between 3 and 8 cm wide, thereby making them unavailable to the blue crab fishery. Infected crabs are castrated and often termed "button crabs." Males appear as miniature adult females, with secondary sexual characteristics transformed to those of a female (Reinhard, 1950, 1956; Adkins, 1972). Diseased crabs are below market size so parasite prevalence is difficult to estimate. Since the parasite removes crabs from the fishery, it causes significant economic losses during epizootics.

SUMMARY

There are several pathogens that cause mortalities in both wild and captive blue crabs. Various factors such as life history, environmental conditions, and water quality act together to influence the degree of disease or parasitism in wild and captive crab populations. Although some methods have been devised to reduce the potential risk of some of these diseases, further research should lead to better control -- especially in captive populations.

ACKNOWLEDGMENTS

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IMPACTS OF INTRODUCED DISEASES, PESTS, AND PREDATORS ON MARINE FISHERIES AND MARINE SYSTEMS

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ABSTRACT

Increasing numbers of transfers and introductions of marine fish and shellfish from their native habitats into new ecosystems are occurring worldwide. With the movement of these exotic species into new environments, there is also the increased risk of introducing associated diseases, pests, and predators. The impacts associated with the movement of marine species are both detrimental and beneficial. Not as well documented as the occurrence of these new arrivals is the impact they and their diseases will have on the recipient native marine species. There is even less information on the impact to the habitats into which they have been introduced. State (prefectural), national, and international efforts now exist to prevent the concomitant introduction of pathogens, parasites, pests, predators, and other biological entities. The Office International des Epizooties (OIE) has identified a number of reportable fish, crustacean, and molluscan diseases that should be tested to prevent their introduction into non-endemic areas. Diseases of oysters have had disastrous effects on oyster populations around the world. Viral diseases of salmonids are an increasing problem in both wild and cultured populations. Recently, four new viral diseases have impacted the international shrimp aquaculture industry. Possible impacts on marine fisheries and systems using specific examples of oyster, fish, and shrimp introductions are: (1) reduced growth, or death of native species; (2) competition with native species; (3) effects on human health; (4) effects on other human activities; (5) habitat destruction.

Key words: invasive species, non-indigenous, diseases, pests

INTRODUCTION

Extremely large numbers of marine organisms, including their diseases, pests, and associated predators, have been and are continuing to be introduced into new marine environments around the world. We know they have an impact on the new habitat and the native species that were there when they arrived, but how is that impact measured? If the species being replaced is hardly known but to a few inquisitive scientists, can we measure that loss?

The striped bass - *Moroni saxatilis* - was introduced to the west coast of the United States and has flourished (Laycock, 1966). To the recreational fisherman, it has been beneficial; to other species of fish that compete for the same time and space, it has been a deadly daily battle.

The green crab - *Carcinus maenas*, introduced from Europe - has plagued the Northeast coast of the United States for most of the last century (MacPhail *et al.*, 1955). It is a voracious consumer of softshell clams *Mya arenaria*, and it was blamed for major losses to the Northeast clamming industry. In Maryland's Ocean City, the fishing boats use the green crab as bait in the tautog fishery. The reported range from Nova Scotia to Maryland may be expanding as new introductions via the "bait" pathway occur. In the past, great efforts were made to control this predatory crustacean. Is that loss any less today, or did we just learn to live with it? Grosholz *et al.* (2000) presented an in-depth review on the impacts of the green crab *Carcinus maenas* on native species in California.

In 1990, the green crab was first reported on the west coast of USA in San Francisco Bay and has since spread rapidly northward to Canada's Vancouver Island and southward to Moro Bay, California (Cohen *et al.*, 1995; Grosholz, 1996). Where and how fast will this extremely invasive species travel? What damage will it do to valuable shellfish beds? How will it compete with other native crab species for food and habitat? We can answer these types of questions and manage the problem only by

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studying the biology of the invader, understanding the types of habitats most likely to support the new organisms, and discerning its interactions with other native species. These answers will provide valuable information to enable scientists and managers to accurately predict or forecast the spread and movement of an invasive species in the environment. This can give us valuable time to develop control measures, educate the public, and help reduce future impact on other ecosystems.

In Japan, eel viral kidney disease (Santo *et al.*, 1981) was introduced with the European eel *Anguilla anguilla*, and has caused widespread mortalities in cultured Japanese eels *Anguilla japonica*. Around the world, there is concern about the introduction of another viral disease, viral hemorrhagic septicemia (VHS) in salmonids. Whether it was introduced or has always infected other reservoir host species seems to be the question being argued today. Sindermann (1990) documents the large number of fish and shellfish diseases that have caused significant losses to the animals they affect. Each of the diseases described poses a potential threat to other populations of unexposed animals.

THE MOLLUSCAN CONNECTION

One of the pioneer oyster biologists, Paul S. Galtsoff, wrote a paper on the "Introduction of Japanese oysters into the United States" (1932). That paper would be as appropriate today for this symposium as it was when he wrote it. He recognized the competition for space with native species, and the possibility of introducing diseases and pests. In 1932, he reported that we had already introduced the eastern oyster drill *Urosalpinx cinereus* and the Japanese drill *Tritonalia japonica* Dunker, to the west coast of USA and the slipper shell *Crepidula fornicata*, a native of the U.S. east coast, had been introduced to the U.S. Pacific coast and Europe.

The oyster industry of the world provides an excellent example of what can go wrong when introducing a non-indigenous oyster species to a new location. Why the oyster? The oyster travels well but never alone. The oyster is home to many organisms, who are probably home to many others. The Pacific oyster is making its way around the world, primarily as an aquacultured species, but sometimes as a hitchhiker. It has become established sometimes as an unwelcome guest, e.g., in Australia (Ayres, 1991) and New Zealand (Dinamani, 1991), where it has replaced the more desirable native oysters. It has also become established and is cultivated as a desirable species on the west coast of USA and Canada (Bourne, 1979; Chew, 1979) but with this oyster introduction also came some less desirable species, e.g., oyster drills and a parasitic copepod.

Diseases are a significant problem facing many species of oysters around the world (Farley, 1992). Such has been the experience of the U.S. east coast. Beginning in the mid-1950s, significant mortalities began in Delaware Bay oyster populations. An organism first identified as MSX - *Haplosporidium nelsoni* (Haskin *et al.*, 1966) - has for the last forty some years continued to kill oysters along the east coast of the United States. Where did it come from; was it always there? Samples of oysters, collected by Victor Loosanoff of the Milford (Connecticut) Fishery Laboratory during the 1930s and 1940s, have failed to show evidence of the existence of this parasite (Kern, unpublished data). Recently, Stokes *et al.* (1995) developed a polymerase chain reaction (PCR) probe. This probe cross-reacts with an identical parasite found in the Pacific oyster from the Republic of Korea (Kern, 1976) and Japan (Friedman *et al.*, 1991; Friedman, 1996; Burreson *et al.*, 2000). It is likely that this parasite was introduced to USA with the numerous attempts to establish the Pacific oyster on the east coast.

The other major disease that affects the U.S. eastern oyster is known as Dermo - *Perkinsus marinus* (Mackin *et al.*, 1950; Levine, 1978). As a group, *Perkinsus* spp. is becoming a major problem around the world. In addition, we know that *P. marinus* was introduced to Hawaii with eastern oysters (Kern *et al.*, 1973). It has the potential to cause great harm if introduced to new oyster populations.

With the introduction of the Pacific oyster to the west coast of USA came the concomitant introduction of its diseases. A bacterial disease called focal necrosis (Sindermann, 1990) and identified as a species of *Nocardia* (Friedman and Hedrick, 1991) continues to cause mortalities under certain conditions of cultivation. Two similar parasites, *Mikrocytos* sp. and *Bonamia* sp., were identified in west coast oysters and may have spread from USA to Europe through the transfer of oysters (Elston *et al.*, 1986; Cigarria and Elston, 1997).

In Europe, several waves of disease have affected oyster production. Alderman (1980) describes several invasions and how oyster diseases were introduced. One of the first losses to the European oyster industry was caused by an iridovirus that infected gill tissue of the Portuguese oyster *Crassostrea angulata*. This species has never recovered and is no longer a significant commercial species. The second wave of oyster disease began in Brittany, France, in the flat oyster *Ostrea edulis* with a disease called Aber disease caused by a parasite identified as *Marteilia refringens* (Grizel *et al.*, 1974). It was soon followed by a microcell-like parasite, *Bonamia ostreae* (Comps *et al.*, 1980; Pichot *et al.*, 1980). Both of these diseases continue to reduce the production of this preferred native oyster from France. Just recently, a new gastropod predator, *Ocinebrellus inornatus*, was reported in the shellfish culture bay of Marennes-Oléron, France (Pigeot *et al.*, 2000). This muricid gastropod originates from the coasts of the Korean Sea and southern Japan. It has become well established and is causing damage to the oyster farms in France.

SHRIMP VIRUS DISEASE IMPACTS

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHN)

The IHHN virus is a small (20-22 nm) single-stranded DNA virus belonging to the parvovirus group. This disease was first described by Lightner *et al.* (1983) in postlarval penaeid shrimp (*Penaeus stylirostris* and *Penaeus vannamei*) isolated from aquaculture facilities in Hawaii. IHHN virus is a highly lethal disease, causing up to 90% mortality in affected populations of susceptible *P. stylirostris*. Some members of the population that survive IHHN infections are apparently carriers, passing on the virus to progeny and other populations (Lightner, 1996). IHHN also has been documented in wild species of shrimp including: *P. occidentalis, P. californiensis, P. monodon, P. semisulcatus*, and *P. japonicus*.

The IHHN virus is widely distributed in aquaculture facilities in both the Americas and throughout Asia. It is assumed to be enzootic in wild penaeids in the Indo-Pacific and Ecuador. In the Americas, IHHN has been found in wild penaeids in Ecuador, western Panama, and western Mexico. Pantoja (1993) and Lightner *et al.* (1992) reported that Mexican blue shrimp (*P. stylirostris*) populations in the Gulf of California plummeted coincident with the onset of IHHN. Beginning with the 1997-1998 season, landings of blue shrimp decreased approximately 1000 tons a year for 4 consecutive years. Shrimp stocks began to recover only after 6 years of decline. This is the best chronological association of disease and wild population effects currently known. The source of the virus is strongly associated with the release of infected cultured shrimp.

Taura Syndrome Virus (TSV)

Taura syndrome virus (TSV) is a small (28-30 nm) single-stranded RNA virus belonging to the picornavirus group (Hasson *et al.*, 1995). The disease, also described as red tail or blackspot disease, was originally reported in mid-1992 in cultured *Penaeus vannamei* near the Taura River, Ecuador. This first outbreak resulted in catastrophic mortalities of 80-90 % of young *P. vannamei*. TSV has been identified in live post-larval shrimp and brood stocks in hatcheries. It has also been identified in seagull feces and the insect water boatman. Numerous shrimp species native to the western hemisphere, including the United States, are susceptible to TSV under experimental conditions. All three species native to the United States (*P. setiferus, P. aztecus*, and *P. duorarum*) have been infected experimentally (Lightner, 1996; Overstreet *et al.*, 1997). Once infected, *P. setiferus* experiences heavy mortalities.

The TSV disease has reportedly spread throughout aquaculture facilities located in the western hemisphere including Peru, Colombia, Honduras, Guatemala, El Salvador, Brazil, and western Mexico (Lightner, 1996). TSV has been documented in wild postlarval and adult *P. vannamei* from nearshore and offshore fisheries in Ecuador, El Salvador, and off the Mexican state of Chiapas near the border of Guatemala. It has also been reported in U.S. aquaculture and hatchery facilities in Hawaii, Florida, Texas, and South Carolina (Lightner, 1996).

Gross signs of the disease are red tails and/or appendages, cuticular necrosis, soft shells, and cuticular black spots. Positive identification of acute but not chronic infections can be made through histological examination. Chronic infections can be diagnosed by bioassay with specific pathogen-free (SPF) *P. vannamei* or by a commercially available gene probe. Definitive diagnostic methods are available and include a gene probe and PCR hybridization. TSV has been identified by bioassay in imported frozen shrimp (Lotz, 1999).

Yellow Head Virus Syndrome (YHV)

Yellow head virus syndrome (YHV) is caused by a small to moderate size (44 x 173 nm) single-stranded RNA rhabdo-like virus. The virus was first reported in aquaculture operations of the tiger prawn shrimp (*P. monodon*) in Thailand in 1992. YHV is widespread in cultured *P. monodon* and is suspected as the causative agent of major production losses of cultured shrimp in Taiwan, Indonesia, China, and the Phillippines in the late 1980s (Lightner, 1996).

The most obvious clinical sign of the disease is the yellow coloration of the shrimp's head. Histological examination reveals generalized necrosis of lymphoid organs, and connective tissues and cuticular epidermis. Cells showing pyknosis and containing cytoplasmic inclusions are abundant. Available diagnostic techniques include histology, electron microscopy, and bioassay. YHV also has been identified by bioassay in frozen shrimp imported to the United States.

Juvenile shrimp are apparently the most vulnerable to YHV infections, although earlier and later stages appear to be somewhat resistant. Thus, juvenile stages of *P. setiferus, P. aztecus*, and *P. duorarum* can be infected experimentally with YHV, although their postlarval stages appear to be resistant (Lightner, 1996). However, all stages of live shrimp in aquaculture, including nauplii, postlarvae, and brood stocks may be carriers of YHV. Asymptomatic YHV carriers were identified in shrimp from Australia, as well as in shrimp showing signs of the white spot syndrome virus (WSSV) disease in Thailand, India, and Texas (Lightner, 1996). YHV has also been found in *P. merguiensis* and *Metapenaeus ensis* in Australia. YHV has been experimentally transferred to *P. vannamei, P. stylirostris*, and *Palaemon styliferus* (mysid shrimp), an

ecologically important species in marine environments. The ability of YHV to infect a number of other genera and species is a warning that YHV could pose a problem to other U.S. marine crustaceans.

By 1994, YHV had also been identified in India, Malaysia, and Indonesia. In November 1995, YHV was found in aquaculture operations in Texas and, in 1996, was tentatively identified in South Carolina aquacultured shrimp.

White Spot Syndrome Virus (WSSV)

The white spot syndrome virus (WSSV) disease, also variously described as red disease, China virus, and shrimp explosive epidemic disease, is caused by a medium size (100-290 nm) double-stranded DNA virus of the non-occluded baculovirus group. It was first identified in 1992-1993 in China and Taiwan (Wang *et al.*, 1995). WSSV has caused mass mortalities reaching 90%+ in several species of shrimp in aquaculture. This virus has been shown to infect a number of other crustacean species (e.g., amphipods, ostracods, swimming crabs, crayfish, copepods, and shore flies), some of which have transmitted the disease into Asian penaeid aquaculture facilities (Lo *et al.*, 1996) The infection of numerous non-shrimp species and other crustaceans raises concerns that these organisms could act as a reservoir, or intermediate host, presenting a possible pathway to infect not only native shrimp, but also other native marine and freshwater species.

All native U.S. species of shrimp are susceptible to WSSV under experimental conditions (Lightner, 1996). WSSV infects and causes disease in many foreign species of shrimp including: *P. monodon, P. semisulcatus, P. merguiensis, P. indicus, P. chinensis, P. penicillatus, and P. japonicus*. Outbreaks were recorded in 1994-1995 in Thailand, India, Japan, and Korea. WSSV is now believed to infect shrimp farms throughout East Asia, Southeast Asia, Indonesia, and India, and was reported in aquaculture operations in Texas in November of 1995 and South Carolina in 1996.

Clinical signs of the disease include a red color to the entire body and appendages along with small subcutaneous white spots. Histological examination reveals prominent intranuclear inclusion bodies in cuticular epithelium, subcutis, and connective tissues. Definitive diagnostic techniques have been developed, and include a gene probe and PCR hybridization. WSSV has been identified by bioassay, gross examination, and PCR in imported frozen shrimp products in retail stores in the United States (Lightner, 1999).

CONCLUSION

The majority, if not all, of the cases of introduced diseases, pests, and parasites cited in this report are the result of an intentional act. This recognition is not new (Galtsoff, 1932; Mann, 1979; Rosenfield and Mann, 1992), yet there is little applied action to stop the continued introduction of non-indigenous species. In their recent article, Lafferty and Kuris (1996) concluded that biological control of marine pests may be possible, but success has not been demonstrated and modifications to existing strategies and expectations will be required.

The Interagency Aquatic Nuisance Species Task Force reported to Congress their "Findings, Conclusions, and Recommendations of the Intentional Introductions Policy Review," developed under the Aquatic Nuisance Species Prevention and Control Act of 1990 and renewed under the National Invasive Species Act of 1996. The International Council for the Exploration of the Sea approved a"Code of Practice to Reduce the Risks of Adverse Effects Arising from Introductions and Transfers of Marine Species, Including the Release of Genetically Modified Organisms." We must learn from the mistakes we make, but we have been making the same ones for almost a century. Currently, the whole subject of invasive species has taken on new momentum.

In February 1999, President William J. Clinton issued Executive Order 13112 and established the National Invasive Species Council. The new council was instructed to prepare a plan to minimize economic and ecological impacts and the harm to animal and human health associated with invasive species. The council is chaired by the Secretaries of Agriculture, Commerce, and the Interior; and includes the Departments of State, Treasury, Defense, Transportation, and the Environmental Protection Agency. The council will develop guidance on invasive species for federal agencies to use in implementing the National Environmental Policy Act. The council will also promote action at local, state (prefectural), tribal, and ecosystem levels, identifying recommendations for international cooperation, facilitating a coordinated network to document and monitor invasive species effects. The council's plan of action includes leadership coordination, facilitating no federal action; utilization of the National Environmental Policy Act as guidance for federal agencies; prevention of intentional and non-intentional introductions; early detection and rapid response; control and management; restoration; international cooperation; research; information management; and education and public awareness.

The introduction of non-indigenous species, their diseases and pests is an issue critical to the aquaculture environment that directly affects the aquaculture industries of all nations.

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CONTROL OF PENAEID ACUTE VIREMIA (PAV) IN SEED PRODUCTION OF *PENAEUS JAPONICUS*

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ABSTRACT

This study aimed to establish control measures for penaid acute viremia (PAV) in the seed production process of *Penaeus japonicus*. In the seed production of the kuruma prawn in Japan, spawners are completely dependent on wild-captured broodstock. The prevalence of PRDV (penaeid rod-shaped DNA virus), which is the causative virus of PAV, was examined in wild-captured brooders by the use of PCR (polymerase chain reaction) from 1996 to 1998. As a result, the highest PRDV value (10.1 %, 96/955) was detected in the ovaries of female prawns, and detected as well in the juveniles of PCR-positive spawners. These results strongly suggested that the infection source of PRDV was wild broodstock. As a control measure, the selection of brooders based on the detection of PRDV from ovaries before spawning was performed in order to prevent the vertical transmission of PRDV, though PAV broke out in juveniles. Furthermore, PRDV was more detectable in the receptaculum seminis of the spawners after spawning. PAV did not occur when egg segregation was performed based on the detection of PRDV from receptaculum seminis of the brooders after spawning. These results indicate that the selection of eggs should be done in the hatchery.

Key words: PAV, WSS, PRDV, *Penaeus japonicus*, seed production, kuruma prawn, broodstock, PCR

INTRODUCTION

The viral disease which occurred in the shrimp farming industry of western Japan in 1993 caused serious mortalities (Nakano *et al.*, 1994). In Japan, this viral disease of *Penaeus japonicus* has been called penaeid acute viremia (PAV), and the causative virus was named penaeid rod-shaped DNA virus (PRDV) (Inouye *et al.*, 1996). In Japan, PAV has damaged the farming industry of other shrimp species as well as the shrimp hatcheries used in sea-farming (Momoyama *et al.*, 1997; Satoh *et al.*, 1999). In southeast Asian countries, PAV has been regarded as the same disease as white spot syndrome (WSS) (Lo *et al.*, 1996:Takahashi *et al.*, 1996), and has been reported in the kuruma prawn, black tiger shrimp *P. monodon*, redtail shrimp *P. penicillatus*, and Chinese prawn *P. chinensis* (Chou *et al.*, 1995; Peng *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Lightner, 1996). This study focused on the epizootiology of this viral disease in both seed production and nursery culture farms from 1996 to 1999. It was found that the elimination of eggs from polymerase chain reaction (PCR) positive spawners was an effective control measure for PAV in seed production.

MATERIALS AND METHODS

Broodstock and collection of eggs

Adult kuruma prawns, which were caught in coastal waters of Kyushu and Shikoku, were purchased from dealers and used as spawners between May and September 1996, and between April and August 1997.

In 1996, 300-600 spawners were transferred into a tank (1.6 kL) filled with sand-filtered seawater. In 1997, 1-7 spawners were installed in a container (10 L) filled with UV (30,000 μ W/m^3) sterilized water. Transport time to Shibushi Station of the Japan Sea-Farming Association (JASFA) was 4-10 h. The water temperature of the tank was kept at 20-25℃ in 1996, while in 1997 it was kept at 15℃, in order to suppress the breeding by spawners. After

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arrival in 1996, spawners were immediately installed into a spawning tank (35 m^3) at 23-27℃ for 1-3 days to induce spawning. In 1997, the ovaries (0.1g) of all spawners were sampled individually by using a disposable syringe (3.0 mL; needle 19 G), and PRDV was detected individually from all the samples by means of PCR. Only spawners having PCR-negative ovaries (5-12 individuals) were installed into a spawning tank at 24-28℃ to induce spawning.

In 1998 and 1999, 5-18 non-biopsied individuals were stocked separately in 0.5 m^3 spawning tanks at 23-25℃ to induce spawning. Receptaculum seminis was sampled for PRDV detection by PCR from the spawners after spawning. Based on the PCR results regarding the receptaculum seminis, the eggs from PCR-negative spawners were used in the following rearing experiments. Fertilized eggs were washed with clean filtered seawater in 1996, and disinfected with povidone-iodine (final concentration was 5 mg /L as active ingredient) for 5 min during 1997-1999. In 1998 and 1999, the eggs from spawners were stocked in rearing tanks (75L), until the end of the process of PRDV detection by means of PCR. Only eggs from PCR-negative spawners were bred in rearing tanks (150-2,500 m^3). Water temperature was kept at a range of 24-28℃.

Seed production

Filtered seawater and sterilized seawater by UV were employed for seed production in 1996 and 1997-1999, respectively. The larvae were fed *Tetraselmis tetrathele*, nauplii of *Artemia salina* and commercial formula feed. In order to detect PRDV from juveniles by PCR, kuruma prawn samples were gathered at egg, nauplius, zoea, mysis, and postlarval stages (egg: 0.05 mg; P1: 1 mg of average body weight; P5: 1.5 mg; P10: 3 mg; P20: 12 mg; P30: 30mg; P40: 100 mg). In each sampling stage from eggs to P10, the sample was a total amount of 0.1 g. From P20 to P40, 30 juveniles were sampled at each sampling. In nursery facilities, the juveniles were collected for PRDV detection at 10-day intervals. These juveniles had been stocked in concrete rearing tanks whose water temperature was maintained between 25 and 28℃.

Detection of PRDV from wild adult broodstocks

Broodstocks were captured in 5 different areas (central Honshu, Shikoku, and Kyushu) of the coastal waters in Japan from July 1996 to April 1998. Samples of hemolymph, stomach, and gonad of these wild female (955 individuals) and male (314 individuals) prawns were submitted to PCR as described later. The average weights of females and males were 78.1-105.5 and 44.7-63.9 g, respectively. Hemolymph was collected by using a syringe (1 mL; needle: 26 G), and 500 μ L was mixed with phosphate buffer saline (PBS: pH 8.0) for PRDV detection by PCR. Stomach and gonad samples were aseptically extracted at a volume of 100 μ g, and stocked at −80℃ until the PCR detection of PRDV.

DNA extraction and detection of PRDV by PCR

The sample composed of hemolymph, stomach, gonad, receptaculum seminis, and juveniles were homogenized and digested with ISOGEN (Japan Gene Co.). Total DNA was extracted using chloroform and ethanol, and two specific primer sets, P1/P2 and P3/P4, were used as reported by Kimura *et al.* (1996). After 30 cycle amplification for each primer set at 93℃ (60 s), 57℃ (90 s), and 72℃ (60 s), amplified products were analyzed by agarose gel electorophoresis. PRDV-infected *P. japonicus* was processed as a positive control.

RESULTS

Occurrence of PAV in seed production

In 1996, PRDV was detected from juveniles in 4 out of 9 seed productions before transportation to nursery facilities, and the juveniles were abandoned (Table 1). The developmental stage in which PRDV was first detected by PCR was eggs, followed by P5 P10, when the water temperature was 22.1 to 29.4℃. In other cases involving 5 seed productions, PRDV was not detected and no PAV occurred until P20. However, PRDV was detected from the juveniles (P29, P51), which were continuously reared in the Shibushi Station after transport to nursery facilities. High rates of mortality occurred in nursery facilities after PRDV detection, with the mortality rate reaching 50-100% during 10 days. Moribund juveniles showed redcoloration, discoloration, and white spots on the carapace. In 1997, the selection of spawners was based on the results of PRDV detection by PCR from ovaries before spawning. As a result of seed production, PRDV was not detected in juveniles in both a total of 9 seed production trials and 17 nursery cultures, and no PAV occurred (Table 2). In seed production experiments in 1997, however, PRDV was detected in juveniles and PAV occurred in 2 rearing trials conducted from July to August (data not shown). PRDV was detected at high rates in the receptaculum seminis of spawned broodstock.

Detection of PRDV from wild adult brooders

The prevalence rate of PRDV showed high values: [ovary (10.1%)] > [stomach (7.3%)] > [hemolymph (5.8%)] in females (Figure. 1). In males, PRDV was detected in the stomach (6.7%) and spermatheca (4.8%), but not in the hemolymph (Figure.1). The seasonal prevalence of PRDV is shown in Figure 2. PRDV was

Table 1. Detection of PRDV in spawners and juveniles in seed production at Shibushi Station of JASFA and occurrence of PAV in nursery culture in 1996

Trial no.	Spawners		Detection of PRDV in seed production *2 (eggs to P20)	Occurrence of PAV	
	Captured date	PCR test *1		In seed production	In nursery culture
1	May 24, 25	−	−	−	− (0/4) *3
2	Jun. 25	−	−	−	− (0/1)
3	Jul. 12	−	−*4 (P51)*4	−	+ (3/3)
4	Jul. 16, 17	−	−	−	− (0/3)
5	Jul. 24	+	−*4 (P29)*4	−	+ (5/5)
6	Sep. 3	+	+ (Eggs)	+ (Abandoned)	Not done
7	Sep. 4	+	+ (Eggs)	+ (Abandoned)	Not done
8	Sep. 12	+	+ (P10)	+ (Abandoned)	Not done
9	Sep. 17	−	+ (P5)	+ (Abandoned)	Not done

*1 Detection of PRDV in stomach cuticular epidermis of spawners after spawning by 2-step PCR.
*2 Detection of PRDV in larvae and postlarvae sampled during seed production. The stage of prawn in parenthesis represents the developmental stage when PRDV was first detected by 2-step PCR.
*3 (Number of cases PAV recorded / conducted).
*4 PRDV was detected later from juveniles which were reared in the hatchery after their siblings were transferred to nursery facilities.

Table 2. Detection of PRDV in spawners and juveniles in seed production at Shibushi Station of JASFA and occurrence of PAV in nursery culture in 1997

Trial no.	Spawners		Detection of PRDV from postlarvae *1	Occurrence of PAV	
	Captured date	PCR test		In seed production	In nursery culture
1	Apr. 11	−	−	−	− (0/1) *2
2	May 12, 13	−	−	−	− (0/1)
3	May 16	−	−	−	− (0/4)
4	May 17	−	−	−	− (0/1)
5	May 12, 13	−	−	−	− (0/2)
6	Jun. 11, 12	−	−	−	− (0/3)
7	Jul. 17	−	−	−	− (0/3)
8	Jul. 19	−	−	−	− (0/1)
9	Jul. 22	−	−	−	− (0/1)

*1 Detection of PRDV in postlarvae just before transportation to nursery culture facility.
*2 (Number of cases PAV recorded/conducted).

detected at an extremely high rate in brooders when captured from summer to autumn (July to December) in 1996, but the prevalence of PRDV was shown to decline gradually, and increased again in summer (July to August) in 1997 and 1998.

Detection of PRDV before and after spawning

The results of detection in the ovary and receptaculum seminis of spawners before and after spawning are shown in Table 3. The prevalence of PRDV in the ovary was 0.9% in 1997, and 0% in 1998 and 1999 before spawning. The values for ovary after spawning were 4.7% in 1997, 0.5% in 1998, and 1.9% in 1999. In the receptaculum seminis, the total prevalence of PRDV before spawning was 5.6% in 1997, 0% in 1998, and 2.1% in 1999, whereas after spawning the values were 33.5, 6.3 and 10.9%, respectively. Thus, PRDV was detected in the receptaculum seminis at a higher rate after spawning than those before spawning, and its prevalence increased rapidly from June on.

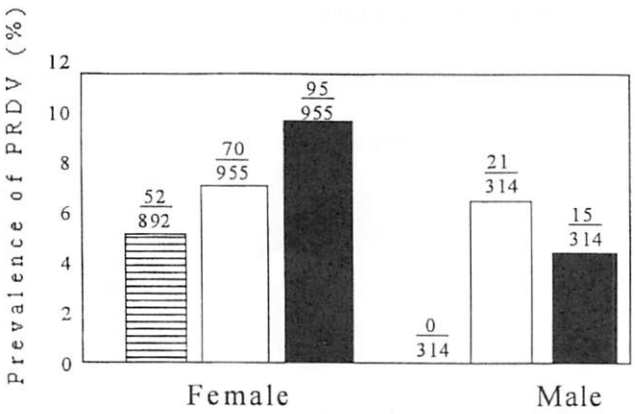

Figure 1. Prevalence of PRDV detected by (2-step) PCR in female broodstock captured in 5 different coastal waters from 1996 to 1998.
Hemolymph Stomach Gonad

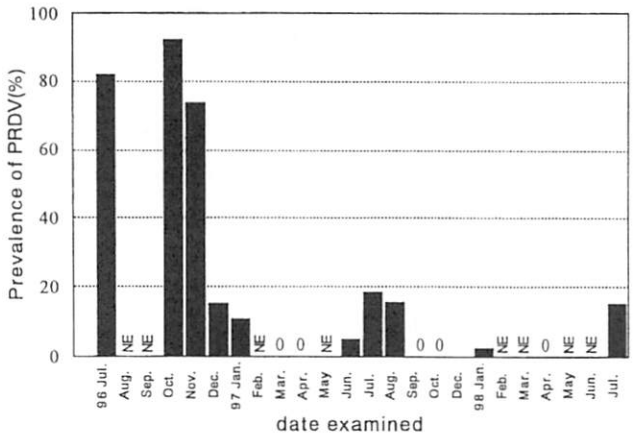

Figure 2. Seasonal prevalence of PRDV in wild female broodstocks of kuruma prawn captured in coastal waters of eastern Kyusyu. NE: Not examined

Table 3. Prevalence fo PRDV in ovary and receptaculum seminis of kuruma prawn spawners by nested PCR before and after spawning during 1997-1998

Date of purchase		Prevalence of PRDV (%)			
		Ovary		Receptaculum seminis	
year	month	Pre-spawning	Post-spawning	Pre-spawning	Post-spawning
1997	Apr.	1.6 (4/248) *1	3.4 (3/ 87)	NE *2	NE
	May	0 (0/ 81)	0 (0/ 40)	NE	NE
	Jun.	0 (0/108)	0 (0/ 37)	0 (0/ 38)	2.3 (2/ 86)
	Jul.	0.7 (2/297)	4.0 (4/101)	8.6 (6/ 70)	39.2 (83/212)
	Aug.	1.3 (3/240)	10.0 (10/100)	5.7 (2/ 35)	52.6 (41/ 78)
	Total in 1997	0.9 (9/974)	4.7 (17/365)	5.6 (8/143)	33.5 (126/376)
1998	Mar.	0 (0/ 3)	0 (0/ 18)	0 (0/ 3)	0 (0/ 18)
	Apr.	0 (0/ 38)	0 (0/587)	0 (0/ 38)	1.4 (8/587)
	May.	0 (0/ 13)	0.9 (1/111)	0 (0/ 13)	0.9 (1/111)
	Jun.	0 (0/ 13)	1.6 (2/122)	0 (0/ 13)	13.9 (17/122)
	Jul.	0 (0/108)	1.4 (2/148)	0 (0/ 21)	24.3 (36/148)
	Total in 1998	0 (0/175)	0.5 (5/986)	0 (0/ 88)	6.3 (62/986)
1999	Mar.	0 (0/ 15)	0 (0/181)	0 (0/ 15)	0 (0/181)
	Apr.	0 (0/ 10)	0 (0/262)	0 (0/ 10)	0 (0/262)
	May.	0 (0/ 5)	0 (0/ 39)	0 (0/ 5)	0 (0/ 39)
	Jun.	0 (0/ 10)	6.8 (5/ 74)	0 (0/ 10)	41.9 (31/ 74)
	Jul.	0 (0/ 7)	0 (0/ 15)	14.3 (1/ 7)	56.1 (37/ 66)
	Total in 1999	0 (0/ 47)	1.9 (12/622)	2.1 (1/ 47)	10.9 (68/622)

*1 (PCR positive / examined).
*2 Not examined.

DISCUSSION

In juveniles which had originated in eggs obtained from spawners, PAV did not occur up to P20. However, PAV was detected in a portion of the juveniles after transportation to nursery culture in 1996. In 1997, after the selection of spawners based on PRDV detection and disinfection of eggs with iodine were employed as PAV control measures during seed production, PRDV was not detected in juveniles nor did PAV occur in either seed production or nursery culture. From these results, the major infection route of PRDV in seed production was considered to be vertical transmission (Satoh *et al.*, 1999).

From the results of PRDV detection by PCR in wild adult kuruma prawns, it was thought that stomach epidermis was not suitable as a target organ for PRDV detection. In a follow-up study, a high prevalence of PRDV was detected in the ovary or receptaculum seminis of spawners. Therefore, it was considered reasonable to detect PRDV from the organ of the spawner that showed the highest

prevalence during the selection of broodstock. It has been found that PRDV was detected in the receptaculum seminis at a higher rate after spawning than in ovaries before spawning (Mushiake *et al.*, 1999). In the present study, it was shown that the selection of eggs obtained from PCRnegative spawners based on PRDV detection from receptaculum seminis after spawning was an effective way of controlling PAV in seed production. Since 1998, PAV has not occurred in the seed production of kuruma prawns at JASFA, showing that these measures have been effective for the past 3 years (Table 4).

The increase in the PRDV detection rates from wild-captured kuruma prawns in summer (July to August) corresponds with the result of *P. monodon* (Lo *et al.*, 1996). It was thought that the increase of WSS virus in broodstocks was probably caused by stress due to multiple spawning between March and September. The possibility that multiple spawning would become an induction factor of virus multiplication in the host has been shown in viral nervous necrosis of the striped jack *Pseudocaranx dentex* (Mushiake *et al.*, 1994). There is no data identifying a similar phenomenon in the kuruma prawn; however, the possibility might exist because the spawning season of the kuruma prawn ranges between April and October and this species repeats copulation and spawning several times in one season.

Table 4. Occurrence of PAV at Shibushi and Kamiura station of JASFA in 1996 to 1999

Year	Selection of spawners *1	Disinfection treatment of eggs	No. of successes / conducted
1996	Not done	Not done	6/14 (42.9%)
1997	From ovary pre-spawning	Iodine	21/23 (91.3%)
1998	From R. S. *2 after spawning	Iodine	11/11 (100%)
1999	From R. S. after spawning	Iodine	16/16 (100%)

*1 Selection based on PCR results.
*2 R. S. : receptaculum seminis.

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JUVENILE OYSTER DISEASE (JOD) AND MANAGEMENT STRATEGIES: A REVIEW

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ABSTRACT

Juvenile oyster disease (JOD), a fatal disease of cultured oysters *Crassostrea virginica* in the northeastern United States, is believed to have been first observed in 1984. By 1990, the disease was found in *C. virginica* cultured in Maine, Massachusetts, Rhode Island, New York, New Hampshire, and Connecticut. Affected oysters exhibit gross characteristics such as the reduction or cessation of growth in previously fast growing juveniles followed by onset of heavy mortalities, development of conchiolin on the inner valve surface(s), mantle recession, formation of shell checks, and occasionally cupping of one valve. Mortalities of 60 to 100% are common and of a rapid onset in first-year oysters. Histological examinations of JOD-infected oysters reveal 2-6 μm singular intracellular inclusion bodies with Feulgen-positive nuclei in vacuoles within healthy mantle epithelium that progress in size and number to form large ulcers in mantle epithelium. No specific organism has been identified as the causative agent. Studies have: shown the disease to be infectious; defined the incubation period; determined the infective agent to be filterable; and evaluated the effect of some environmental influences. Effective management strategies include spawning oysters early, from January to April, to allow juveniles to grow above the size where they are most heavily affected by the disease before the onset of JOD, and developing strains of oysters resistant to JOD.

Key words: juvenile oyster disease, JOD, *Crassostrea virginica*, oyster mortality, disease resistance

INTRODUCTION

Mortalities of hatchery-produced juvenile oysters, *Crassostrea virginica*, from a previously unrecognized disease syndrome were documented in Maine oysters reared in Great Bay, New Hampshire, as early as 1984 (Farley and Lewis, 1997). By 1990, similar mortalities were occurring in *C. virginica* cultured in Maine (ME), Massachusetts (MA), Rhode Island (RI), New York (NY), New Hampshire (NH), and Connecticut (CT) (Rask, 1990, 1992; Bricelj *et al.*, 1992; Relyea, 1992; Farley and Lewis, 1993, 1994b; Davis and Barber, 1994; Ford, 1994; Barber *et al.*, 1996; Lewis *et al.*, 1996c; Farley and Lewis, 1997). The disease has become known as juvenile oyster disease (JOD) and has a well-documented disease syndrome (Bricelj *et al.*, 1992; Davis and Barber, 1994; Lewis *et al.*, 1996c).

The disease was first believed to be caused by a genetic, nutritional, toxin, or environmental problem (Bricelj *et al.*, 1992; Rask, 1992; Lewis, 1993), but studies found no evidence to support these hypotheses (Farley and Lewis, 1994a; Lewis *et al.*, 1996c; Paillard *et al.*, 1996). Several different strains of brood stock exposed to JOD in early field studies were shown to be equally susceptible and suffered heavy mortalities (Ford *et al.*, 1993a, b; Ford, 1994; Lewis and Farley, 1995; Lewis *et al.*, 1996c). Disease onset and mortalities occur despite adequate food availability and histological examinations show no evidence of nutritional deficiency (Bricelj *et al.*, 1992; Wikfors and Smolowitz, 1994; Ford and Tripp, 1996). Unlike disease caused by toxins or environmental conditions, JOD is specific to cultured oysters. Other shellfish or marine life occupying waters with JOD-infected eastern oysters are not adversely affected by JOD (Bricelj *et al.*, 1992; Davis and Barber, 1994; Farley and Lewis, 1994a, b). Although JOD rarely involves wild oysters, Ford (1994) reported low chronic mortalities and some conchiolin deposits among 1991 wild set CT oysters deployed in bags in Oyster Bay and Long Island Sound, NY. A low prevalence of conchiolinous shell lesions (not quantified) was also reported in wild oysters from Long Island Sound, CT.

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Examination of 1992 wild set juvenile oysters from Long Island Sound, NY and Thames River, CT showed $<4\%$ prevalence of gross signs of JOD (Farley and Lewis, 1994a, b). No evidence of conchiolin or other signs of JOD were found in 1993 wild set juveniles from Long Island Sound or MA. Histological evidence from infected juveniles showed no involvement of gills, palps, or digestive system tissues that would likely be exposed to insult by toxins.

Field and laboratory research has provided significant documentation of the disease, but no etiologic agent has been identified. The two most strongly supported hypotheses suggest protistan or bacterial involvement (Bricelj *et al.*, 1992; Farley and Lewis, 1993; Small, 1995, 1997; Lee *et al.*, 1996a; Lewis *et al.*, 1996c; Paillard *et al.*, 1996; Boettcher *et al.*, 1999, 2000). Lewis and Farley (1993a) and Farley and Lewis (1995) have shown this disease to affect juvenile oysters from Long Island, NY north to ME. Experimental studies have shown the yet unidentified cause of JOD to be a filterable, temperature- and salinity-sensitive biological agent that is readily transmissible in laboratory controlled experiments using JOD-infected oysters, inoculation of extracts from JOD-infected oysters, or material filtered from the water column at JOD-affected sites (Lewis, 1993; Ford *et al.*, 1993a; Farley and Lewis, 1994a; Lewis *et al.*, 1995, 1996c; Paillard *et al.*, 1996; Farley and Lewis, 1997). Also, the disease agent is susceptible to the antibiotics erythromycin, norfloxacin, and sulfadimethoxine-ormetoprim (Farley and Lewis, 1994a; Lewis *et al.*, 1996c; Boettcher *et al.*, 1999, 2000).

The disease consists of multiple gross and histological characteristics that collectively comprise the JOD syndrome (Bricelj *et al.*, 1992; Farley and Lewis, 1993; Lewis, 1993; Lewis and Farley, 1993b; Davis and Barber, 1994; Lewis *et al.*, 1996c; Farley and Lewis, 1997). The first indication of disease is a slowing or cessation of growth in previously fast growing juveniles 5-30 mm in shell length followed by an acute onset of mortality that peaks rapidly, then declines in 4-6 wk from onset. Cumulative mortalities of 60% are not unusual and may reach 90-100%. Oysters $>$ 25-30 mm may show a high prevalence of disease, but are much less likely to die (Bricelj *et al.*, 1992; Farley and Lewis, 1993; Davis and Barber, 1994; Ford, 1994; Ford and Paillard, 1994; Farley and Lewis, 1994a; Ford and Tripp, 1996; Lewis *et al.*, 1996c).

Upon gross examination of oysters, the disease is strongly associated with the production of a golden or light olive-green conchiolinous deposit on the internal shell surface (Bricelj *et al.*, 1992; Davis and Barber, 1994; Farley and Lewis, 1994a, b; Lewis and Farley, 1995; Ford and Tripp, 1996; Lewis *et al.*, 1996c). Oyster diseases, noxious agents, irritants, and shell injury can cause conchiolin deposition in oysters as a normal defense response and repair mechanism (Orton, 1924; Galtsoff, 1964; Farley, 1968; Carriker, 1996). However, the conchiolinous shell lesions associated with JOD are unique (Lewis and Farley, 1993a; Farley and Lewis, 1994a) in that they are more prevalent and extensive, and vary from a thin partial film to a thick wall that completely encapsulates the infected oyster in advanced stages. This shell lesion is a key gross diagnostic feature for the disease (Lewis, 1993; Farley and Lewis, 1994a; Ford, 1994; Paillard *et al.*, 1996). Conchiolin often covers most of the inner shell of both valves, including the area under the adductor muscle as it migrates during growth. Mantle recession (mantle retraction) is common in many oyster diseases and is frequently observed as JOD progresses. Abnormal shell growth causing conspicuous shell checks (growth pauses appearing as distinct ridges, growth bands, or in extreme cases similar to the overhang of a fingernail) are found on the external shell of some surviving oysters (Lewis and Farley, 1993a; Farley and Lewis, 1994b; Ford and Tripp, 1996; Lewis *et al.*, 1996c). These checks coincide with cessation of growth at disease onset and later regrowth of new shell in survivors. The regrowth of new shell may give the appearance of a small oyster atop a larger oyster and the oyster may completely change the direction of growth. Occasional abnormal deep cupping of the left valve or separation of the adductor muscle from the shell, occurs as the muscle migrates over conchiolin-covered shell.

The presence of mantle lesions are common in JOD-infected oysters upon histological examination (Bricelj *et al.*, 1992; Farley and Lewis, 1993, 1994a, 1997; Lewis *et al.*, 1996c) and statistically correlate with oyster mortalities (Farley and Lewis, 1994a; Ford, 1994). Small 2-6 μ m singular intracellular inclusion bodies (coccoid bodies) with 1-3 μ m Feulgen-positive nuclei, found focally in healthy mantle epithelium of infected oysters, are routinely observed (Bricelj *et al.*, 1992; Lewis, 1993; Lewis *et al.*, 1996c; Farley and Lewis, 1997). By mortality onset, inclusions progress in size and number and ultimately lead to lysis of mantle tissue, sloughing of necrotic host tissue (and possibly necrotic cells of the disease agent), and formation of ulcerations in the mantle epithelium (Bricelj *et al.*, 1992; Lewis *et al.*, 1996c; Farley and Lewis, unpublished data). Hemocytic infiltration occurs in the epithelium and underlying connective tissue of the mantle.

TRANSMISSION STUDIES AND ENVIRONMENTAL INFLUENCES

Relationship of water temperature and JOD transmission

While JOD is not caused by elevated water temperatures, laboratory and field experiments showed the incubation period for JOD to develop in *C. virginica* depends strongly on water temperatures (Farley and Lewis, 1993; Lewis, 1993; Ford, 1994; Barber *et al.*, 1996; Lewis *et al.*,

1996c; Davis and Barber, 1999). The onset of mortalities in late June to late July in the Long Island, NY area and in late July through August in ME is associated with a sustainable rise in water temperature above 18-20 ℃ (Farley and Lewis, 1993; Lewis, 1993; Davis and Barber, 1994; Ford, 1994).

Field studies carried out at Frank M. Flower and Sons, Inc. (FMF) in Bayville, NY and the Cornell Cooperative Extension facility (CCE) in Cedar Beach, Southold, NY showed the disease occurred 3-8 wk after water temperatures exceeded and maintained 20℃ (Farley and Lewis, 1993; Lewis 1993). Oyster mortalities continued throughout the summer and early autumn with some growers reporting low levels of mortality throughout the winter (Rask, 1990; Lewis, 1993; Farley and Lewis, 1997). In Damariscotta River, ME, Davis and Barber (1994) observed the first mortalities to occur 6-7 wk after growing waters exceeded 20℃. Oysters from a low temperature site (17-12 ℃) in ME experienced higher cumulative mortalities (87% vs. 72%) compared to a second site (24-17℃), even though mortality onset was delayed by 3 wk at the colder site (Davis and Barber, 1999). Mortalities at the first site occurred from late August until October. Mortalities at both sites occurred after water temperatures had peaked at or above 19℃.

Early laboratory studies (Lewis, 1993; Lewis *et al.*, 1996a, b, c) showed JOD to be an infectious disease that consistently resulted in the expression of the typical JOD syndrome and incubation period as seen in natural infections at similar water temperatures. Uninfected hatchery-reared oysters (n = 175 oysters/aquarium) showed an acute onset of JOD mortalities 3-7 wk after exposure to JOD-infected oysters in aquaria with recirculating water held at constant temperatures of 18, 20, 22, and 24℃ and 26 ppt salinity. Timing of disease onset varied inversely with water temperature. Cumulative mortality in aquarium-challenged oysters ranged from 41% (18℃) to 74% (24℃). Similar incubation periods were observed in field studies of experimental and cultured oysters grown in JOD-infected waters. Consistent with natural infections, histological examination of experimentally infected oysters routinely revealed lesions with intracellular bodies in mantle tissue as described by Farley and Lewis (1993, 1994a). No control oysters revealed these mantle lesions. Evidence of bacterial lesions or viral infection were not found in diseased oysters from experimental studies (Lewis *et al.*, 1996c).

Paillard *et al.* (1996) transmitted JOD by inoculation of extracts prepared from JOD-infected oysters directly into the pallial cavity of challenge oysters. After inoculation, oysters were held out of water, forcing the oyster to stay closed and maximizing contact with the inoculate. A one-time inoculation of supernatant extract into replicate groups of 40-60 mm experimental oysters (n = 40 oysters each) that had been notched at the ventral edge produced conchiolinous lesions in 11 and 15% of oysters after 10 days. With two inoculations, the prevalence of conchiolin increased to 40 and 45%. Cumulative mortality after 4 wk was 9%.

The potential of transmitting JOD to other species of aquaculture-reared oysters was investigated using *Ostrea edulis* and *C. gigas*. Preliminary experiments (Lewis and Farley, 1996, 1997b) showed that juveniles of both species exposed to JOD suffered mortalities that mimic JOD, but the JOD syndrome was not duplicated. Also, *O. edulis* cultured near JOD-infected *C. virginica* in ME showed no indications of JOD (Barber *et al.*, 1998). Juvenile clams *Mercenaria mercenaria* or *Mya arenaria* grown in JOD-infected waters did not show evidence of disease (Farley and Lewis, 1994b). Juvenile *M. mercenaria*, grown near JOD-infected oysters, were used in transmission experiments to determine if the clam may filter and retain the JOD agent and thus serve as an infectious source for juvenile oysters. The clams did not transmit the disease to uninfected juvenile *C. virginica* in laboratory experiments (Lewis and Farley, 1997b).

Relationship of salinity and JOD development

Natural infections occur in salinities exceeding 25 ppt. Results of field and laboratory transmission studies and exposure of JOD-infected oysters at constant salinities show that salinities below 18 ppt inhibit or prevent disease transmission rather than increase the incubation period for the disease to develop fully (Lewis *et al.*, 1996b, c).

Laboratory transmission of JOD to uninfected hatchery-reared oysters (n = 200 oysters/aquarium) readily occurred at salinities of 18 ppt and higher, with cumulative mortalities up to 75% after 4-6 wk of exposure (Lewis *et al.*, 1996c). At the end of 6 wk, cumulative mortalities at salinities of 10 and 15 ppt were 16% and 14%, respectively. No conchiolinous shell lesions were observed in oysters exposed at 10 or 15 ppt and mortalities were similar to that of control oysters.

In a 6-wk salinity controlled experiment, oysters naturally infected by JOD (n = 200 oysters/aquarium) showed decreased mortality (40-51%) when exposed to salinities less than 18 ppt (Lewis *et al.*, 1995, 1996c). Cumulative mortalities in oysters held at salinities of 18-30 ppt ranged from 60-85%. Oysters were exposed at 23-27℃ and 18-24℃.

In 1993, FMF oysters from a single spawn (A2M) exposed to JOD were placed upriver where salinity fluctuates from 10 ppt to approximately 0 ppt on tidal cycle. No mortality was observed in the low salinity group while mortalities in the group grown in the traditional high salinity nursery ranged from 27-53% (n = 50 oysters examined/sample) during the same 6-wk period (Farley and Lewis, 1994a). Histological examinations of oysters reared at low salinity showed a 7% or less prevalence of

intracellular inclusion bodies and ulcerations in mantle epithelium and a 7-10% prevalence of conchiolinous shell lesions. This compares to a prevalence of 55% for intracellular inclusion bodies, 49% mantle ulcerations, and 76% conchiolinous shell lesions in high salinity oysters. Growth was slightly less in oysters held at low salinity. Gross and histological data from these and other oysters showed a significant correlation in days of exposure, hemocyte infiltration, mantle ulcerations, intracellular inclusion bodies, and conchiolinous shell lesions with mortality from JOD (Farley and Lewis, 1994a,b). Likewise, Ford (1994) reported mantle lesions were highly correlated with mortality occurring 2 wk after observed lesions.

Shellfish growers introduced oysters from a New England hatchery affected by JOD into at least six sites in Chesapeake Bay in 1993 (Farley and Lewis, 1995; Lewis and Farley, 1996). Heavy mortalities were found in samples of juveniles collected from one site. According to the grower, only juveniles introduced from New England experienced mortalities. In this case, no conchiolin was evident in dead oysters. At another site, however, the JOD agent apparently survived exposure to salinities of 5 ppt for 7 mo after importation of juvenile oysters from the same New England hatchery (Farley and Lewis, 1995; Lewis and Farley, 1995; Lewis *et al.*, 1995). A sample of these oysters (n = 57), quarantined without exposure to JOD-infected oysters or water, experienced heavy mortalities (79%) and developed conchiolinous shell lesions (46%) characteristic of JOD after being placed in room temperature aquaria at 26 ppt salinity for 2 mo. Studies (Farley and Lewis, 1994a) suggest that salinities below 18 ppt, typical in most of the Maryland portion of Chesapeake Bay, are not favorable for proliferation and transmission of the disease agent.

FILTRATION STUDIES

Laboratory and field studies show that the JOD agent is filterable. Exposure of cultured oysters in the hatchery to ambient filtered water at a JOD-endemic site (FMF) showed mortalities were reduced but not eliminated and disease onset delayed by 1-2 wk by passing water through a 25-μm bag filter compared to filtration at 50 μm, no filtration, and traditional nursery growout (Farley and Lewis, 1993; Ford *et al.*, 1993a; Lewis and Farley, 1995; Lewis *et al.*, 1995).

Repeated laboratory transmission experiments using material filtered from the water at FMF showed the typical incubation period and expression of the JOD syndrome. Material captured from pumping ambient water (11,000 to 14,000 L) sequentially through various combinations of bag filters (including 100, 50, 25, 10, 5, and 1 μm pore size filters) was used as an inoculum to infect oysters (n = 200 oysters/aquarium) during spring, summer, and autumn when water temperatures ranged from 15 to 28℃. Transmission was consistently evident at all particle size levels above 5 μm and occasionally at the 1-5 μm level. From experiments to date, the disease agent has not passed a 1-μm-mesh bag filter (Lewis *et al.*, 1996a, c). Studies showed JOD infections could be initiated by filtered material from FMF as early as May when the ambient water temperature was 15℃. Mortalities (4%) with typical conchiolinous shell lesions (75%) were observed only in oysters exposed to material held by a 10-μm-mesh bag filter, approximate particle sizes 10-25 μm. Unlike the high levels of *Vibrio* spp. in the inocula, apparently the reservoir of JOD-infective particles present at that time of year was sufficient only to initiate light infections with low mortalities (Lewis and Farley, 1995). Mean total *Vibrio* counts from triplicate samples of inocula ranged from 10,000-1,100,000 colony forming units (CFU) / ml, depending on the level of filtration.

THERAPEUTANTS

Therapeutic treatments for JOD were investigated by Lewis *et al.* (1996c) and Boettcher *et al.* (1999), primarily for the purpose of yielding information on the identity of the JOD causative agent, not to identify a treatment for JOD. In addition, naturally infected oysters that had just begun to experience JOD mortality were used to test the effect of medications on disease progression.

Lewis and Farley (1994) and Lewis *et al.* (1996c) investigated the effects of over-the-counter saltwater aquarium medications Maracyn (erythromycin), Maracyn-Two (minocycline), CopperSafe, and Ick Guard (triethylene glycol, Victoria green, nitomersol, and acriflavine) on JOD-infected oysters (n = 200 oysters/treatment). Erythromycin had the greatest effect in reducing cumulative mortality to 9% compared to 65% in infected control oysters and worked most effectively with retreatment every 3 wk. Maracyn is sold to eradicate gram-positive bacteria but erythromycin has also been found to be lethal to protists, such as ciliates, by disrupting mitochondrial activity (Lewis *et al.*, 1996c). Treatment with an ectoparasitic medication (CopperSafe) reduced mortalities to 69% compared to 85% in untreated control oysters. Maracyn-Two and Ick Guard had no beneficial effects.

Oysters grown in floating trays in the Damariscotta River, ME that were immersed in norfloxacin or sulfadimethoxine-ormetoprim solutions (n = 500 oysters/treatment) 3 h/wk showed a delay in onset of JOD and reduced mortality (Boettcher *et al.*, 1999). Cumulative mortalities in treated oysters were 55 and 67%, respectively, compared to 81% in control oysters treated with filter-sterilized seawater. The antibiotics were

selected because of their broad spectrum activity against bacteria, being bactericidal by inhibition of DNA unwinding and folic acid synthesis, and selective toxicity for bacteria.

EVIDENCE FOR A PROTISTAN OR BACTERIAL AGENT

Numerous causes have been suggested for JOD, but none proven. Currently, a protist or bacterium is viewed as the most likely disease agent.

While most investigators seem to agree on basic histological findings from JOD-infected oysters, interpretations vary. Histological studies have shown the presence of 2-6 μm intracellular bodies (coccoid bodies) in normal mantle epithelium and mantle lesions of JOD-infected oysters (Bricelj *et al.*, 1992; Farley and Lewis, 1993, 1994a; Lewis, 1993; Lewis *et al.* 1996c; Farley and Lewis, 1997). Some investigators suggest the bodies to be protistan parasites mixed with degenerate host cells (Farley and Lewis, 1993, 1994a, b). More specifically, the presence of small, paired, dense staining, Feulgen-positive nuclei, one larger than the other, in many of the intracellular bodies was suggestive of ciliate macro and micronuclei among degenerate host tissue to some investigators (Farley and Lewis, 1993, 1994a, b), while others suggest them to be solely degenerate oyster tissue (Bricelj *et al.*, 1992). Mortalities occur within 1 to 2 wk of an increased intensity of the intracellular bodies in mantle tissue (Farley and Lewis, 1994a) and the formation of mantle lesions (Ford, 1994), then taper off 1 wk after a decrease in intensity of the intracellular bodies (Farley and Lewis, 1994a). Mantle lesions were shown to statistically correlate with oyster mortalities (Farley and Lewis, 1994a; Ford, 1994). Gross and histological data also showed a significant correlation in days of exposure, hemocyte infiltration, mantle ulcerations, intracellular inclusion bodies, and conchiolinous shell lesions with mortality from JOD (Farley and Lewis, 1994a, b; Ford, 1994).

Bricelj *et al.* (1992) and Ford *et al.* (1993a) reported bacteria were present in some lesions where mantle epithelium had been eroded and were common in moribund oysters. In oysters collected weekly or biweekly from time of deployment through periods of peak mortality, a small percentage of histological slides showed bacteria and these were exclusively from oysters with mantle lesions (Ford, 1994). Finding bacteria only in low prevalence and in oysters with advanced stages of disease suggests the bacteria may be secondary invaders.

Despite numerous attempts to identify a bacterial etiology for JOD, evidence for such cause remains inconclusive (Lee *et al.*, 1994, 1996a; Lewis *et al.*, 1996c; Boettcher *et al.*, 1999, 2000). In oysters grown in stacked trays suspended near the water surface, Lee *et al.* (1996a, b) found concentrations of *Vibrio* spp. increased one order of magnitude in tissues of nursery oysters 2 wk prior to the onset of mortalities. Vibrio concentrations in water and debris samples did not correlate with the oyster mortalities, but vibrio concentrations in the sediments rose significantly before oyster mortalities and decreased after. Nine of 200 *Vibrio* spp. isolated from oyster tissues 1-3 wk prior to onset of heavy mortality caused mortality when inoculated into naive oysters, but failed to produce typical conchiolinous shell lesions (Lee *et al.*, 1996a). Similarly, *Vibrio* spp. isolated from JOD-infected oysters caused mortalities when added to aquaria water in high concentrations, but no single isolate produced clear or consistent signs of JOD (Lee *et al.*, 1996b)

Bacterial suspensions of 10 predominant strains isolated from oysters showing signs of JOD were prepared from 72-h pure cultures grown on marine agar and injected directly into the pallial cavity of oysters (Paillard *et al.*, 1996). None of the isolates produced strong or consistent JOD-like infections. Mortality was variable, ranging from 8-33%, and occurred mostly in the first week after inoculation.

Lewis *et al.* (1996c) cultured bacteria from: shellfish growing waters; infected oysters throughout the Northeast; and inocula used to transmit JOD in the laboratory. Results showed no association between isolated bacteria and JOD. Using trypticase soy broth with 2% sodium chloride, marine broth, alkaline peptone water, trypticase soy agar with 2% sodium chloride, thiosulfate-citrate-bile sucrose agar, and marine agar culture media, 17 *Vibrio* spp. and 32 species of other bacteria were isolated nearly equally from control and infected samples (Lewis and Farley, 1997b; Lewis *et al.*, 1997). Further, bacterial lesions were not found in tissues of JOD-infected oysters upon examination by light or electron microscopy (Farley and Lewis, 1994a; Lewis *et al.*, 1996c).

Boettcher *et al.* (1999, 2000) identified an α-proteobacteria of the *Roseobacter* group (designated CVSP) as the numerically dominant bacterium in JOD-infected oysters from ME. However, the bacterium was not isolated before the onset of mortalities which appears to be inconsistent with histological data (Farley and Lewis, 1994a; Ford, 1994) showing pathology (intracellular bodies and mantle lesions) 1-2 wk before mortality onset. As with other bacterial studies, injection of cultured CVSP bacterium (approximately 3.3×10^7 cells/oyster) into the mantle cavity of naive oysters (n = 121) held at 20-22°C caused mortalities without consistent signs of JOD. Significant mortalities were not observed until 12 wk post-injection, well past the expected incubation period for JOD at the exposed water temperature. Also, conchiolinous shell lesions were not observed in dead oysters. Challenge experiments exposing naive oysters in aquaria (n = 200 oysters/aquarium) containing synthetic water (19-21°C) inoculated with the α-proteobacteria failed to cause

mortality above 0.5% after 5 mo. Aquaria were inoculated with a final concentration of either 10^3 or 10^6 CFU/ml of bacteria after a 1-wk acclimation period and three times weekly thereafter for 5 mo.

MANAGEMENT STRATEGIES

Effective management strategies must minimize losses to disease and prevent transmission of disease outside an affected area. JOD is transmitted readily when susceptible seed oysters are placed in waters containing the infectious agent. Also, survivors of JOD mortalities and oysters grown in JOD-infected waters may act as disease carriers (Lewis *et al.*, 1996c). To date, the most effective management strategies for JOD include timing the spawning and growout of juveniles to avoid summer periods when the effects of JOD are most severe, and developing disease-resistant strains of oysters.

Spawning

The first successful management strategy for JOD was based on the demonstrated relationship between JOD, water temperature, and oyster size. By spawning oysters earlier in the year, juveniles reach the 25-30 mm size refuge before the annual onset of JOD (Davis and Barber, 1994; Farley and Lewis, 1994a; Relyea, 1994, 1995; Barber *et al.*, 1996). This technique reduces, but does not eliminate, impacts of JOD.

Results of the early spawning strategy in the Long Island, NY region showed increased survival more than offset increased energy and labor costs to spawn and rear juveniles. Now, spawning primarily during January through April with less production in May and June is a common practice. A late window of opportunity also exists for growers in the Damariscotta, ME area. Deployment of juveniles in late August avoids peak JOD activity (Davis and Barber, 1994, 1999), but benefits of this strategy must be balanced against increased costs and risks of overwintering mortality (Barber *et al.*, 1996; Farley and Lewis, 1997). Late spawns were unsuccessful in preventing JOD losses in NY, presumably due to warmer water temperatures there in the late summer and early autumn (Lewis and Farley, 1997a).

Development of JOD-resistant strains

Mortalities from JOD continued at high levels in the early 1990s and caused some growers and hatcheries to go out of business. In 1986, scientists from the University of ME in collaboration with industry growers on the Damariscotta River, ME began a program to develop a fast growing oyster adapted to ME growing conditions (Barber *et al.*, 1998; Davis and Barber, 1999). The program was based on the selection of the largest 20% (total weight) of oysters as brood stock after 18 mo of growth. After JOD emerged in the Damariscotta River, the selection criteria automatically included survival of JOD events (Davis *et al.*, 1995; Davis and Barber, 1999). The goal was to produce progeny that would quickly grow to sizes >25-30 mm before disease onset.

Resistance was ultimately successful in reducing mortalities ($n = 500^+$ oysters/tray; stocking density = 2000 oysters/m^2) from 96% in susceptible oysters to 11% in resistant oysters during 1996 field trials (Barber *et al.*, 1998). Studies of Davis and Barber (1999) showed mortalities of 72, 87, and 82% in control oysters compared to 61, 26, and 43%, respectively, in selected oysters depending on strain and exposure site in the Damariscotta River in the 1994 and 1995 growing seasons ($n = 500$ oysters/growing cage).

In 1993, scientists of the NOAA Cooperative Oxford Laboratory initiated a collaborative study with FMF and CCE to develop strains of oysters resistant to JOD (Farley and Lewis, 1994a). One- to 2-year-old JOD survivors were selected as brood stock based on survival of JOD and the presence of multiple exaggerated shell checks. Results of earlier work demonstrated an association of shell checks in JOD survivors with multiple bouts and recovery from JOD (Farley and Lewis, 1994a; Farley *et al.*, 1995).

Since the FMF and CCE JOD-resistant strains were developed and field tested on Long Island in 1993 and 1994, mortalities from JOD have been reduced tremendously, harvest of oysters has returned to normal, and in most recent years survival of eyed larvae to 25 mm is at record levels (D. Relyea, FMF, Bayville, NY, personal communication). Initial resistant stocks showed up to 7 times better survival in 1994 than naive brood stocks (Farley *et al.*, 1995). In 1995, survival was 7 to 25 times better in resistant strains and mortalities decreased from 75% to 0-3% in size culled seed (<20 mm) at FMF on Long Island, NY (Farley *et al.*, 1996; Lewis and Farley, 1996). Cumulative mortality of unculled resistant oysters in field trials at six sites ($n = 2000$ oysters / strain; 3 strains) on Long Island was less than 15% (typically 0-6%) over an 11-wk period compared to 43 to 75% in susceptible control oysters. Resistance was found to develop in the first spawning and not improve with successive generations up through the F_3 (Farley *et al.*, 1997a, b).

Effects of water flow and stocking density on JOD infections

Reports suggest increased water flow reduces, but does not eliminate, JOD. Ford (1994) reported decreased mortalities in oysters reared in floats when bottom mesh size was increased from 1 to 9 mm, thus increasing water exchange or flow rates through the floats. Thus, blockage of water flow through culture bags by fouling organisms, a

normal summertime occurrence, should be reduced or eliminated by periodic cleaning to minimize JOD-induced mortalities.

Water flow experiments of Rivara and Czyzyk (1995a, b) utilizing flow rates of 4, 20, and 40 L/min and oyster stocking densities of 1, 6, and 12 L/silo showed increased water flow to be more important for growth and preventing JOD mortalities than stocking density. By wk 4 of the experiment, low flow silos averaged 60% mortality compared to 33% in high flow silos (n = 100 oysters/treatment examined each week). Oysters were from the same spawning batch and of the same size at the beginning of the 5-wk experiment at CCE. Rivara and Czyzyk (1995b) recommend that growers utilizing an upweller system employ a medium flow/medium stocking density combination for growing oysters in areas affected by JOD; however, silos can be highly stocked with oysters if relatively high flow rates (0.03 L/min/cm^2 silo screen area) are used (Rivara and Czyzyk, 1995a).

Bricelj *et al.* (1992) reported less mortality in oysters when stocked at a density 11-66% of traditional commercial growout by FMF. Mortalities in the small cohort were reduced from approximately 62 to 34% and 54 to 39% in the large cohort. Stocking density of oysters throughout the growing season varied according to oyster size.

Filtration

Filtration may be another effective management technique in certain situations. Hatcheries typically filter incoming water as a standard procedure; however, the level of filtration varies according to the hatchery. Some hatcheries filter at the 25, 10, or 5 μm level with bag filters, each of which has been shown to pass JOD-infective particles (Lewis *et al.*, 1996c). Bag filters are not precise in pore size, and overly used filters may allow particles to pass through that otherwise would not due to pressure and pore size distortion. For unknown reasons, JOD may not always affect every batch of juvenile oysters spawned, or even groups within a spawning batch, at some hatcheries each year. Likewise, some growers may not be as strongly affected every year by the disease. Cyclic variation of disease pressures and variance in the degree of water filtration may be partially responsible for some batches of hatchery seed oysters showing indications of JOD infection while others do not. Further, if the size of the infective particle changes due to differences in life stages or by infection of a larger carrier organism, as happens with suctorial ciliates, this may change the level of filtration necessary during different seasons. Lewis *et al.* (1996a, c) demonstrated a well-maintained sequential filtration of incoming hatchery water to 1 μm can be effective in eliminating the infectious agent. The more effective the filtration, the less likely the agent will pass to oysters grown in hatcheries or upwellers.

CONCLUSION

JOD continues to occur in oysters in the northeastern United States; however, the prevalence has been greatly reduced since the early 1990s by the development of JOD-resistant oysters and implementation of other management strategies. Identification of the disease agent, however, remains elusive.

Slight evidence of JOD ($<$1 - 2% prevalence of conchiolin and even less mortality) is still found most years at FMF in resistant oysters, but only in juveniles spawned late in the year (D. Relyea, personal communication). However, in the autumn of 2000, FMF experienced mortalities $>$ 50%, but only in juveniles from a June spawn. On eastern Long Island, NY as much as approximately 15% of juveniles from some growers may show evidence of disease, but mortalities have been limited to approximately 5% or less (G. Rivara, Cornell Cooperative Extension, Southold, NY, personal communication). A RI grower reports no mortalities in recent years from JOD (R. Rheault, Moonstone Oysters, Narragansett, RI, personal communication) since switching suppliers of juvenile oysters and utilizing a floating upweller system (FLUPSY) to rear juveniles $>$ 25-30 mm before deploying them for growout. In ME, some growers reported significant losses ($>$50%) during the summer of 2000 in areas of the Damariscotta and New Meadows rivers (D. Morris, University of Maine Cooperative Extension, Darling Center, Walpole, ME, personal communication).

As studies have shown, JOD is an infectious disease that can be naturally transmitted to oysters by exposure to infected oysters or infected growing waters. Therefore, oysters spawned and reared in waters contaminated by the JOD organism may become infected and act as carriers of the disease when moved to new locations. With development of JOD-resistant strains of oysters, gross signs of JOD may be reduced to the extent that it is difficult to assay an oyster or batch of oysters for JOD without histology. Histology likewise may be insufficient for diagnosing JOD in early cases when infection prevalence and intensity are low or when environmental conditions are unfavorable at the time of sampling to allow full development of the disease (Farley and Lewis, 1994a; Paillard *et al.*, 1996). This potentially poses a problem for importers and shippers of oysters from JOD-endemic areas since oysters that survive JOD events may show only limited signs of infection. As with any shellfish movement, great consideration should be made before moving shellfish from JOD-infected growing waters to unaffected areas. Specific guidelines adopted by the International Council for the Exploration of the Sea (ICES) can help reduce risks of disease associated with the transfer of marine shellfish to

non-contiguous bodies of water (ICES, 1995). In part, these recommendations include the: quarantine of imported stocks in an approved system to allow adequate evaluation of the health status of the stock; introduction of the first generation progeny into the natural environment only after no disease agents or parasites become evident in the first generation progeny; and treatment of effluents from the quarantine facility to kill all living organisms present in the effluent.

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PATHOGENESIS OF *PERKINSUS* SPP. IN BIVALVE MOLLUSCS

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ABSTRACT

Two distinct species of *Perkinsus* were isolated from infected softshell clams (*Mya arenaria*), one closely related to *P. marinus* and the other recently described as a new species, *P. chesapeaki*. The histopathology of *Perkinsus* species infections varies among infected hosts. For example, *P. marinus* in oysters causes large abscesses. The parasites destroy the digestive tract epithelium, infiltrating protozoal cells multiply within hemocytes, and infections become rapidly systemic. Damage to the oyster is caused mainly by lysis of affected tissues due to extracellular products, mainly serine proteases, secreted by the parasite. *Perkinsus* species in softshell clams typically localize in the gills and are encapsulated by hemocytes within discrete cysts. Large abscesses may be found in most tissues in severe infections and may interfere with normal metabolic processes. The widespread tissue lysis often observed in oysters heavily infected with *P. marinus* has not been observed in softshell clams infected with *Perkinsus* species. The ability of pathogenic protozoa to damage tissue depends partially upon the production of lytic enzymes and adhesion molecules collectively known as virulence factors. We identified extracellular proteins (ECP) secreted by the two softshell clam *Perkinsus* species and compared the ECP of the two isolates with those of an oyster-derived isolate of *P. marinus*, P-1. The proteolytic activities of softshell clam *P. marinus* were found to be serine protease in nature, similar to those of P-1; however, P-1 showed significantly higher proteolytic activity. Conversely, *P. chesapeaki* ECP lacked proteolytic activities and was more highly lipolytic. The difference in enzyme activities observed among the three *Perkinsus* species provides a possible explanation for differences observed in *Perkinsus* species infections in clams and oysters. These studies provide important insights into invertebrate cellular defense mechanisms and host-parasite interactions which will enhance the development of improved disease prevention and management strategies for cultured and feral bivalve molluscs.

Key words: softshell clam (*Mya arenaria*), *Perkinsus*, pathogenesis, protease, zoospores

INTRODUCTION

The integrity of the marine ecosystem depends, to a great extent, on the health of its fauna and flora. As filter-feeders, bivalve molluscs safeguard water quality in estuaries and minimize effects of severe environmental fluctuations. Over the past decades, the depletion of major marine and estuarine fisheries worldwide has alarmed the public, scientists, and governmental agencies. Natural and anthropogenic factors such as habitat degradation, increased fishing pressure, and infectious disease have been claimed as the causes for this decline. As aquaculture operations accelerate worldwide in the exploration for new alternatives to overburdened natural resources, the need exists to better understand diseases of marine organisms. The current gaps in our knowledge on pathogens and mechanisms of disease initiation and progression in invertebrates, including molluscan species, impede efforts by the scientific community and resource managers to identify the full dimension of the problem and, consequently, to recover and restore collapsing fisheries.

Of all known molluscan diseases, those caused by *Perkinsus* species occupy a central position (Perkins, 1993). Prevalent in warmer coastal waters, *Perkinsus* species have been associated with serious losses of ecologically and economically important resources worldwide. *Perkinsus marinus* is a widespread, serious pathogen of eastern oysters *Crassostrea virginica* and causes severe epizootics throughout

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the Gulf of Mexico, the Chesapeake and Delaware bays, and recently, northern states on the U.S. Atlantic coast (Mackin *et al.*, 1950; Perkins, 1976; Burreson *et al.*, 1994; Burreson and Ragone Calvo, 1996; Ford, 1996; Krantz and Jordan, 1996). Severe losses of other commercially important bivalves have been associated with other *Perkinsus* species including *P. olseni* of the black lip abalone *Haliotis ruber* (Lester and Davis, 1981), *P. atlanticus* of the Portuguese clam *Ruditapes decussatus* (Azevedo, 1989) in Spain (Figueras *et al.*, 1992; Navas *et al.*, 1992) and Italy (Da Ros and Canzonier, 1986), and *P. qugwadi* of cultured Japanese scallops *Patinopecten yessoensis* (Blackbourn *et al.*, 1998). A pathogenic *Perkinsus* species was associated with mortalities in bay scallops (*Argopecten irradians*) (McGladdery *et al.*, 1991). This review summarizes a series of investigations leading to the identification of a new *Perkinsus species, P. chesapeaki*, isolated from the softshell clam *Mya arenaria* of the Chesapeake Bay (McLaughlin *et al.*, 2000b).

PERKINSUS SPP. IN *MYA ARENARIA*, 1954-1999

Mya arenaria

Despite the ecologic and economic importance of softshell clams, surprisingly little is known about their basic biology in general and diseases in particular. This is alarming since the devastation in the eastern oyster fishery in the Chesapeake Bay is primarily associated with the pathogens *Haplosporidium nelsoni* and *P. marinus* (Mackin *et al.*, 1950; Mackin, 1960; Levine, 1978). The softshell clam currently occupies a wide geographical range in the northern hemisphere, on both coasts of the Atlantic as well as on the Pacific east coast. Softshell clams are among the dominating species in some areas of Europe including the Baltic Sea and the west coast of Sweden, and on the Atlantic coast of North America in Nova Scotia and New England (Strasser, 1999). Softshell clams live in subtidal soft bottom communities in the Chesapeake Bay and became an important economic resource with the development of the hydraulic escalator dredge in the early 1950s (Hanks, 1963). Unfortunately, the annual landings of softshell clams have steadily declined in the Chesapeake Bay from over 8.2 million kg of meats in 1951 to less than 45,000 kg in 1998.

Epizootiology

As early as 1954, it was reported that softshell clams in the Chesapeake Bay host *Perkinsus* species infections (Andrews, 1955). The parasite, however, was rare in occurrence and low in intensity particularly when compared with eastern oysters infected with *P. marinus*. Surveys performed over two decades (1969-1989) by the Maryland Department of Natural Resources (MDDNR), Oxford, Maryland, showed no evidence of *Perkinsus* infections in softshell clam populations of the upper Chesapeake Bay based upon examination of over 3500 histology preparations (S.V. Otto, MDDNR, Oxford, MD, personal communication). In the late 1980s, scientists detected *Perkinsus*-like hypnospores in histologic tissues of softshell clams with some regularity, and suspected softshell clam *Perkinsus* infections were more common than previously thought (McLaughlin *et al.*, 1995). A fear arose that these infections may have contributed, directly or indirectly, to the steady decline in landings observed in this important commercial species since 1962 (NMFS, 1996). Therefore, a need arose to thoroughly investigate *Perkinsus* species infections in the softshell clam.

In the 1990s, a series of studies was initiated to investigate the biology and pathogenesis of *Perkinsus* species infections in softshell clams of the Chesapeake Bay. First, routine epizootiological surveys of over 1700 softshell clams in 57 samples (~30 clams/sample) were performed at 18 sites in the upper portion of the Chesapeake Bay from 1990-1998 (McLaughlin and Faisal, 2000). Prevalences of *Perkinsus* infections in softshell clams were determined by utilizing a rapid and sensitive assay developed by Ray (1952). Rectal tissues were excised from the softshell clams and incubated in Ray's fluid thioglycolate medium (RFTM) prepared with seawater and fortified with antibiotics (Mackin *et al.*, 1950; Ray, 1966). *Perkinsus* cells enlarged in the medium, developed cell walls that stained blue-black with Lugol's iodine, and were enumerated using a modification of the rating system by Mackin (1962) to assign weighted prevalences to varying intensities of infection. A 7% (114/1705) prevalence of *Perkinsus* species was observed in softshell clams examined from 1990-1998. Peak prevalences of softshell clam *Perkinsus* species were typically observed in late summer and fall when salinities and temperatures were highest. Intensities of infections based upon rectal thioglycolate assays ranged primarily from extremely light to heavy (stages 1-5) with extreme cases (stages 6 and 7) observed in less than 1% (9/1705) of softshell clams examined. The maximum weighted prevalences observed were 1.17 at the Piney Point site in September 1992 and 1.57 at Cedar Point in August 1995. This apparent elevation in *Perkinsus* infections in softshell clams paralleled increased prevalences and range extensions of *P. marinus* infections in the eastern oyster in the Chesapeake Bay (Burreson and Ragone Calvo, 1996). The increased occurrence of *Perkinsus* infections in oysters and softshell clams was concurrent with high salinities due to successive droughts during the 1980s, coincidental mild winter temperatures, and movement of *Perkinsus*-infected oysters. Further, prevalences of softshell clam *Perkinsus* species followed infection patterns observed in oysters with peaks generally occurring in late summer or early fall when

temperatures and salinities were highest.

Pathology

Examination of histology preparations from softshell clams collected during the epizootiological surveys of 1990-1998 revealed the gills to be a major site of *Perkinsus* species infection (McLaughlin *et al.*, 1995). In the eastern oyster, light infections of *P. marinus* are usually limited to the digestive epithelium and mantle connective tissues (Mackin, 1951). Hence, rectal and mantle tissues have been utilized in RFTM assays for routine diagnosis of *P. marinus* infection (Ray, 1966). As infection intensity increases, any major organ of the oyster may be used in RFTM assay due to the spread of protozoal cells to almost every tissue (Mackin, 1951). Moreover, the systemic nature of *P. marinus* infection affords the use of a less invasive assay which utilizes hemolymph withdrawn from live animals and incubated in RFTM (Gauthier and Fisher, 1990). Conversely, the gills were found to be the major site of infection by *P. atlanticus* in the carpet shell *R. decussatus* (Azevedo, 1989) and Manila clams *R. philippinarum* (Rodriguez and Navas, 1995). This led Rodriguez and Navas (1995) to recommend the use of gill tissue for routine RFTM diagnosis of *P. atlanticus* infection in these clam species.

Similarly, a study to compare diagnostic assays for detection of *Perkinsus* species in the softshell clam was performed (McLaughlin and Faisal, 1999). The gills, labial palps, rectal tissue, and hemolymph from 90 softshell clams were compared for their suitability in thioglycolate diagnosis of *Perkinsus* species infection. Paraffin tissue sections were also examined to determine the utility of histological diagnoses as compared to RFTM assays. Examination of hemolymph and tissue samples following incubation in RFTM revealed the presence of spherical blue-black cells typical for hypnospores of *Perkinsus* species. In general, the prevalence of *Perkinsus* species was higher in gill and palp tissues than in rectal tissues. As the intensity of *Perkinsus* species infection increases in the softshell clam, the number of positive rectal tissue samples also increases, and in advanced infections is parallel in intensity to those of the gills and palps. Thus, the use of both gill and rectal tissues in separate RFTM assays was recommended by McLaughlin and Faisal (1999) to provide information on infection intensity and progression in each clam. Finally, diagnosis of *Perkinsus* species by RFTM assay revealed more positive samples than the histological examination of stained tissue sections.

Histopathology

Although histology was found to be the least effective for detecting light *Perkinsus* species infections in softshell clams, examination of infected tissues provides important insights into host-parasite interactions (McLaughlin and Faisal, 1999). McLaughlin and Faisal (1998a) described the most common histopathological alterations associated with *Perkinsus* species infections in a survey of 240 softshell clams collected from the Chesapeake Bay. Examination of Mayer's hematoxylin and eosin (MHE) stained tissue sections revealed a 12% (28/240) prevalence of *Perkinsus* infections. Most of the infected clams had light parasite burdens concentrated predominately in gill tissue. Moderate infections were observed in ~20% of infected clams and were characterized by increased parasitism in the gills and low to moderate numbers of parasites in other tissues. The number and size of cysts increased as infection intensity increased. Only a few of the clams examined were considered to be severely infected, with most tissues being affected. In these clams, large lesions associated with *Perkinsus* cells were observed in gills, digestive gland, gonads, kidneys, muscle tissue and other major organs. Massive parasite aggregates and fused lamellae typical of advanced *Perkinsus* species infections may interfere with respiration. Similarly, large abscesses observed in most major organs in more advanced infections may interfere in other normal physiologic processes such as reproduction and growth. Despite a strong host reaction, a progressive nature to the infections was apparent in some clams where numbers of parasites and sizes of abscesses seemed to increase proportionally. While the number of advanced cases was substantially less than early infections, there remains a possibility that clams with advanced infections may die as a direct result of *Perkinsus* infection or indirectly due to secondary invaders or other stressors, and thereby escape detection. Results of the study suggested a more significant effect by *Perkinsus* species parasites on softshell clam tissues than previously surmised.

The pathological alterations reported by McLaughlin and Faisal (1998a) in *Perkinsus* species-infected softshell clams are different from those described for eastern oysters infected with *P. marinus*, where the infection runs an aggressive systemic course (Mackin, 1951). *Perkinsus marinus* destroys the digestive epithelium of eastern oysters, and infiltrating protozoal cells are able to multiply within hemocytes and become rapidly systemic. Damage to the host is caused mainly by lysis of affected tissues due to products secreted by the parasite (Mackin, 1951; La Peyre and Faisal, 1995a; La Peyre *et al.*, 1996; Faisal *et al.*, 1999). Most important is the reduced growth rate observed in infected oysters (Ray and Chandler, 1955; Paynter, 1996). Widespread tissue lysis observed in oysters heavily infected with *P. marinus* was not observed in softshell clams infected with *Perkinsus* species (McLaughlin and Faisal, 1998a). However, a clear tissue response was provoked in softshell clams with infiltrating granulocytic hemocytes playing a major role. The dissemination of parasites in softshell clams appears to be delayed by their encapsulation within discrete cysts.

In vitro propagation

The three studies outlined above provided evidence that *Perkinsus* infections could indeed be a significant problem in softshell clam populations of the Chesapeake Bay. Therefore, a need arose to further identify softshell clam *Perkinsus* species and to determine any relationships among *P. marinus* of the eastern oyster and other *Perkinsus* species infecting molluscs worldwide. First, however, a sufficient supply of the parasites was needed to conduct the biochemical and phylogenetic evaluations required to identify and characterize softshell clam *Perkinsus* species. The ability to grow Perkinsus species in continuous culture in the laboratory is a result of a relatively recent breakthrough (La Peyre *et al.*, 1993; La Peyre and Faisal, 1995b). Continuous cultures were established from pericardial tissue of infected oysters using media closely resembling bivalve plasma composition (La Peyre, 1996). Soon after, Kleinschuster and Swink (1993) and Gauthier and Vasta (1993) also initiated continuous cultures of the oyster parasite. Utilizing similar growth mediums, other *Perkinsus* species have since been isolated and cloned in culture including *P. atlanticus* (Ordas and Figueras, 1998) and an uncertain *Perkinsus* species found in *Macoma balthica* (Kleinschuster *et al.*, 1994).

The culture of *Perkinsus* species from softshell clams was achieved by utilizing the media and methodology developed for *P. marinus* (McLaughlin and Faisal, 1998b). Surprisingly, two morphologically distinct species of *Perkinsus* were isolated and cloned from softshell clams. The two continuously-propagated axenic cultures of *Perkinsus* species were obtained from clams collected from the Chester River in the Chesapeake Bay in the fall of 1996. Cultures were initiated from hemolymph (H-49) and gill tissue (G-117) of softshell clams diagnosed with advanced infections. The characteristic morphology, enlargement in RFTM, and division patterns clearly demonstrated that G-117 and H-49 isolates from softshell clams possessed characteristic features of *Perkinsus* species. However, morphological and cultural characteristics also provided evidence that H-49 and G-117 were non-identical *Perkinsus* species. Except for their larger size, H-49 cells resembled the oyster pathogen *P. marinus*, and divided by schizogony (Mackin *et al.*, 1950; Mackin, 1951). Conversely, G-117 cells were morphologically distinct from H-49 cells and both vegetative (trophozoites and schizonts) and zoosporulation stages were present in the same culture flasks. Life cycle stages exhibited by isolate G-117 were similar to a *Perkinsus* species isolated from the Baltic clam *Macoma balthica* (Perkins, 1968; Valiulis and Mackin, 1969; Kleinschuster *et al.*, 1994) and to those described for *P. atlanticus* (Auzoux-Bordenave *et al.*, 1995). The H-49 isolate was later identified as *P. marinus* and G-117 as a new species, *P. chesapeaki* (McLaughlin *et al.*, 2000b).

Potential virulence factors

Differences in tissue pathology of softshell clams infected with *Perkinsus* species and eastern oysters infected with *P. marinus* depend upon the parasite's ability to invade and damage host tissues and upon the host's defense mechanisms. The ability of pathogenic protozoa to damage tissue depends, at least partially, upon the production of lytic enzymes and adhesion molecules collectively known as virulence factors (McKerrow *et al.*, 1993; Michalski *et al.*, 1994). In this context, previous studies have shown that the pathogenicity of *P. marinus* depends on the expression of extracellular proteins (ECP), including highly potent serine proteases (La Peyre and Faisal, 1995a; La Peyre *et al.*, 1995; Faisal *et al.*, 1999). *Perkinsus marinus* proteases hampered oyster hemocyte functions *in vitro* (Garreis *et al.*, 1996; Tall *et al.*, 1999) and favored the propagation of *P. marinus* within the host (La Peyre *et al.*, 1996).

The *in vitro* production and release of ECP by the two softshell clam G-117 *P. chesapeaki* and H-49 *P. marinus* isolates were analyzed and compared to the P-1 isolate of oyster *P. marinus* previously characterized (La Peyre *et al.*, 1993; La Peyre and Faisal 1995a). Biochemical characterization of softshell clam *P. marinus* H-49 and *P. chesapeaki* G-117 isolates showed distinct differences in enzyme activities (McLaughlin *et al.*, 2000a). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining revealed the presence of protein bands in cell-free culture supernatants of H-49 and G-117. The ECP profiles of G-117, H-49, and P-1 shared common bands; however, each isolate possessed a unique banding pattern. The profile of *P. chesapeaki* G-117 ECP was distinct from either softshell clam *P. marinus* H-49 or oyster *P. marinus* P-1. The H-49 ECP profile was similar but not identical to P-1. The H-49 samples demonstrated proteolytic activity by the hydrolysis of gelatin incorporated into SDS gels. This hydrolytic activity was more pronounced in P-1 samples and completely absent in *P. chesapeaki* G-117 samples. The lack of any proteolytic activity by *P. chesapeaki* G-117 ECP suggests the isolate may depend on other mechanisms for its virulence. The proteolytic enzymes of softshell clam *P. marinus* H-49 were further shown to contain serine proteases similar to those reported in oyster *P. marinus* P-1. API-Zym analyses revealed additional proteolytic activity (chymotrypsin) in H-49 which was lacking in G-117 ECP. The ECP of G-117 however had higher lipolytic activities than either H-49 or P-1 ECP. In contrast, lipolytic activities of H-49 ECP were greater than P-1. The role of lipolytic enzymes in virulence is unknown; however, lipases may be involved in energy metabolism by the parasite, penetration of host cell membranes, or may be essential for dissolving host-formed capsules. The differences in the electrophoretic patterns of the three isolates are most likely associated with virulence factors and host specificity.

Genetic analyses

Molecular studies were conducted to confirm the identity of the two isolates as *Perkinsus* species and to further distinguish the H-49 and G-117 isolates as distinct species (Kotob *et al.*, 1999a, b). Genetic variation between the two clam isolates complemented differences observed in morphology, life cycles, and biochemical products. The small subunit ribosomal ribonucleic acid (SSU rRNA) genes of H-49 and G-117 were amplified utilizing *Taq* polymerase and two primers designed from the published sequence of SSU rRNA gene of *P. marinus*. Each polymerase chain reaction (PCR) fragment was inserted into a thymidine adenosine (TA) cloning vector and transformed into *Escherichia coli*. Sequencing of PCR products SSU rRNA loci of G-117 and H-49 indicated that the sizes of these genes were within the previously reported ones for other *Perkinsus* species and confirmed the relatedness of G-117 and H-49 to the genus *Perkinsus* (Kotob *et al.*, 1999a). Additional molecular studies using more variable regions of the ribosomal RNA loci were needed to more closely characterize the two softshell clam isolates. The internal transcribed spacer (ITS-1 and ITS-2) regions and the 5.8S ribosomal RNA genes of G-117 and H-49 were cloned and sequenced (Kotob *et al.*, 1999b). Sequence similarities of ITS-1 and ITS-2 genes were high between *P. marinus* and H-49; however, sequence similarities for the same genes were dissimilar for G-117 and six other *Perkinsus* species. Genetic differences among *Perkinsus* species determined by pairwise comparison of ITS-1, ITS-2 and 5.8S rRNA sequences also revealed similarities between H-49 and *P. marinus*, and showed G-117 to be distinct from other *Perkinsus* species. Kotob *et al.* (1999b) concluded from the genetic similarity between isolate H-49 and *P. marinus* that they both belonged to the same species. Finally, this study not only supported the inclusion of G-117 and H-49 in the genus *Perkinsus* but supported evidence of a closer relationship between *Perkinsus* and dinoflagellates than apicomplexans (Fong *et al.*, 1993; Goggin and Barker, 1993; Siddall *et al.*, 1997; Reece *et al.*, 1997).

Description of zoospores

Descriptions of new *Perkinsus* species have been premised upon morphology of life cycle states, ultrastructure of zoospores, the host affected, and host responses to infection (Mackin *et al.*, 1950; Perkins, 1976; Lester and Davis, 1981; Azevedo, 1989; Blackbourn *et al.*, 1998). Zoospore morphology in particular has been thought important in differentiating *Perkinsus* species (Perkins, 1976; Azevedo, 1989; Lester and Davis, 1981; and Blackbourn *et al.*, 1998). The morphological, biochemical, and genetic characterization studies of H-49 and G-117 softshell clam *Perkinsus* species isolates identified H-49 as *P. marinus* and provided evidence of G-117 as a new species. The final step in this series of investigations was to examine the ultrastructure of G-117 zoospores. Zoosporulation studies were performed on gill-associated *Perkinsus* species incubated in RFTM for 5 days and then for 24 h in sterile JL-ODRP (La Peyre *et al.*, 1993) growth medium. Macerated tissues or culture media were transferred to flasks containing sterile seawater and flasks were examined daily until zoosporulation occurred. Zoosporulation preparations were fixed for electron microscopy and ultrastructural features were compared to other described species. Ultrastructure characteristics of the zoospores supported the inclusion of the gill-associated G-117 isolate in *Perkinsus*. For example, the zoospores are biflagellated with flagella of unequal length. A unilateral array of hair-like structures is present on the anterior flagellum. The presence of a simplified apical complex and associated structures provides further evidence to support including G-117 in the *Perkinsus* group. Differences observed in the ultrastructure of the zoospore stage distinguished this isolate from other described species. The shape and size of the zoospore body, length of flagella, and ultrastructure characteristics of the nucleus and mitochondria combined with morphological, biochemical, and genetic data supported the designation of G-117 as a new species, namely *Perkinsus chesapeaki* (McLaughlin *et al.*, 2000b).

CONCLUSIONS

In summary, results of these investigations have provided insights into the phenotypical, biochemical, and molecular aspects of members of the genus *Perkinsus*, known to cause devastating losses of bivalve molluscs worldwide. The genetic similarity between isolate H-49 and *P. marinus* extends the demonstrated host range of *P. marinus* to include both the eastern oyster and the softshell clam. Further, these studies provided the first evidence that a coinfection by two *Perkinsus* species can exist in one molluscan species. It is, however, difficult to distinguish among *Perkinsus* species in host tissues or RFTM assays. Monoclonal antibodies are needed to replace the current polyclonal antibodies being used to detect *Perkinsus* species in tissues and in the water column. Additional molecular tools, such as PCR assays, may provide more effective distinction of *Perkinsus* species and greater insight into host parasite interactions. Further, the development of biochemical tools based upon enzyme products may provide a rapid and effective way to detect and differentiate various *Perkinsus* species. Continuous cultures and gene sequences of two previously unidentified species of *Perkinsus* species produced in this study will be valuable resources for future investigations related to species identification and pathogenic mechanisms of this important parasite.

The decline of naturally reproducing shellfish

populations worldwide is attributed to natural and anthropogenic factors including pollution, overfishing, and disease. Intensive and extensive shellfish culture operations have rapidly expanded in the last decade to meet the demand for seafood by consumers and to support stock enhancement programs. Early recognition and diagnosis of disease problems is a critical component of managing shellfish aquaculture systems. These studies provide important insights into invertebrate cellular defense mechanisms and host-parasite interactions which will enhance the development of improved disease prevention and management strategies for cultured and feral bivalve molluscs.

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THE NOAA/DOC AQUACULTURE INITIATIVE

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The advancements and accomplishments of the new NOAA/DOC aquaculture effort will be summarized in terms of purpose, vision, goals and funding. A short summary of funded projects will be provided and an overview of the program management strategy will be presented. The NOAA Strategic Plan for sustainable fisheries, which includes a strong aquaculture component, will be described and suggestions made as to how to connect with ongoing international programs like the UJNR Aquaculture.

AQUATIC ANIMAL HEALTH : THE ROLE OF DIAGNOSTIC LABORATORIES AND ADVANCED DIAGNOSTIC TOOLS

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The aquaculture industry is driven by demand for finfish and shellfish products and limited by the availability of natural stocks. The industry relies on intensive culture practices in which disease management is the foremost limitation and is often a leading cause of economic loss and aquaculture failures. The University of Connecticut, Department of Pathobiology and Veterinary Science is an established diagnostic laboratory with expertise in aquatic animal health, diagnostics and research. Strengths exist in the disciplines of pathology, toxicology, parasitology, microbiology, immunology, molecular biology and vaccinology. The following are representative cases of department's role in diagnostic medicine, applied research, and the development of advanced diagnostic methods.

***Case I: Development of a multiplex PCR for detection of Haplosporidium nelsoni* (MSX), *Haplosporidium costale* (SSO), and *Perkinsus marinus* (Dermo) in the eastern Oyster, *Crassostrea virginica*.** The monitoring of cultured oyster populations for pathogens is presently infrequent due to a dependence on traditional, time consuming, diagnostic assays. A multiplex polymerase chain reaction (MPCR) has been developed which rapidly detects the protozoan parasites, *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale* (SSO), which infect the cultured oyster, *Crassostrea virginica*. Conventional diagnostic methods (histopathology and Ray/Mackin fluid thioglycollate assay) for *H. nelsoni, H. costale* and *P. marinus* respectively were compared to the MPCR. Ninety-one adult oysters were collected and subjected to all three assays. The Ray/Mackin assay detected *P. marinus* infections in 64% of oysters and MPCR revealed infections in 80%. Histological examination detected 40% of oysters infected with *Haplosporidium* plasmodia. The MPCR was able to differentiate between the two *Haplosporidium* plasmodia, detecting 10% of oysters infected only with *H. nelsoni*, 40% with only *H. costale*, and 35% with mixed infections of *H. nelsoni* and *H. costale*. These results indicate the MPCR is a more sensitive assay for the detection of *P. marinus* and is able to detect and differentiate between the two *Haplosporidium* species. The MPCR is useful at low infection intensity, by being able to detect pathogen DNA at concentrations as low a 10 fg, for *H. nelsoni* and 1pg for both *H. costale* and *P. marinus*.

Case II: Epizootiology, pathology and molecular characterization of a myxosporean associated with parasitic encephalitis of farmed Atlantic salmon (Salmo salar) in Ireland. During seasonal epizootics of disease and mass mortality in the summers of 1992, 1993 and 1994 on a sea-farm in Ireland, Atlantic salmon smolts suffered from an encephalitis associated with an unclassified parasite 6-8 weeks after transfer to sea. In order to monitor disease, determine the onset and anatomic distribution of parasites and encephalitis, and to identify and fully characterize the pathogen, smolts were observed and necropsied from affected sites at 1-3 day intervals for 2 months after transfer to sea netpens. Clinical disease was characterized by circling or gyrating swimming, inappropriate postures in the water column, stacking on pen floors, and periods of apparent unconsciousness. Foci of parasites with and without attendant encephalitis were detected in histologic sections of brain and spinal cord 1-2 weeks before clinical signs; the parasite was detected as early as 26 days post-introduction. The parasite's ultrastructure was consistent with a histozoic presporogonic multicellular developmental stage of a myxosporean, located between axons. No mature spores were identified. In the absence of detectable sporogony, PCR, Southern blot hybridization, DNA sequencing and in situ hybridization were used in concert to characterize the parasite. The parasite is a neurotropic species of the genus Myxobolus, with sequences identical to those of *M. cerebralis*.

Case III: Assessment of the Cause and Extent of Morbidity and Mortality of American Lobsters (*Homarus americanus*) in Long Island Sound. Mortalities of the American lobster, *Homarus americanus*, in Long Island Sound have severely increased. As a result, the U.S. Department of Commerce has declared the

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fishery a disaster, and the regional lobster industry has been critically damaged. Necropsies were performed on individual lobsters collected from Long Island Sound. Gross and histopathologic examination, hematology, microbiology, virology, parasitology and toxicology were performed on affected lobster. Gross observations of'sick' lobsters included lethargic/limp behavior, discoloration of meat, viscera and hemolymph, coagulopathy and hemocytopenia. Bacteriologic findings included isolation of *Vibrio* spp. and spirochetes but no common pathogen. No *Aerococcus* spp. have been isolated. Toxicology included tests for metals, chlorinated pesticides and polychlorinated biphenyl's, and polycyclic aromatic hydrocarbons including malathion. Toxicology has been unremarkable. Histologic examination of all organ systems and associated tissues revealed a systemic inflammatory disease affecting multiple tissues, but primarily the nervous system. Associated with lesions was a protozoan parasite morphologically characterized as an amoeba, tentatively a paramoeba species. Presently, the immediate cause of death is presumed to be the paramoebiasis. Investigations are focused on the parasite and identified climatic and anthropogenic stressors to determine whether the parasite is the primary cause of mortalities and which physiologic stressors are contributing factors. In addition, molecular probes and new diagnostic methods and tools are being developed to assess the health of the lobster and better define the molecular systematics of the newly identified protozoan parasite and other pathogens of the lobster.

APOPTOSIS OF HOST CELLS INDUCED BY M2 GENE PRODUCT OF IHN VIRAL GENOME

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Infectious hematopoietic necrosis (IHN) virus causes salmonid hosts to damage hematopoietic organs and, therefore, to bring about high mortality expressing severe anemic gill and kidney as a typical sign. Since we found that IHN virus-infected rainbow trout demonstrated apoptotic response to circulating lymphocytes detected by DNA fragmentation and TUNEL (terminal nucleotidyl transferase-mediated deoxyUTP nick end labeling), this apoptosis was suggested to be important in the pathology of IHN. We, first of all, attempted to focus our attention on M2 gene having shorter RNA sequence chosen out of genomic genes (G, L, M1, M2, N and NV genes), to investigate IHN viral protein responsible for the apoptosis. M2 gene DNA, produced from IHN viral RNA by reverse transcription (RT) -PCR amplification, was ligated into E. coli plasmid vector. Following the insertion of M2 gene DNA to the vector, recombinant M2 protein was prepared from E. coli transformant. When lymphocytes and cultured cells were incubated with the M2 protein, ca. 200 base pair-ladder was determined. Further, when anti-M2 protein rabbit polyclonal IgG was supplemented with the incubation, DNA ladder formation was strong inhibited. The action of M2 protein was a kind of nonspecific nuclease because of digestion of salmon testis DNA into nucleotide oligomers. The M2 gene, therefore, could be termed 'nuclease gene'. Thus, IHN viral RNA genome included a nuclease gene as a tool for virulence to injure chromosomal DNA of the host tissue cells. These findings claim that the M2 gene product implicated in the apoptosis of host cells should be incorporated as a major mechanism into the pathology of IHN. The administration of the M2 protein by immersion, nevertheless, showed no vaccine effect on rainbow trout alevin infected with IHN virus.

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PROBIOTIC APPROACH TO ENHANCE HEALTH OF HATCHERY PRODUCED SHELLFISH SEED

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Bacterial diseases of intensively cultured larval and juvenile shellfish cause significant losses in hatcheries and nurseries. In addition, chronic bacterial infections are a significant cause of bivalve seed losses post-planting. From commercial hatchery case histories, a number virulent juvenile oyster bacterial pathogens have been isolated, characterized and pathogenicity confirmed by challenge procedures.

Prevention and control strategies for bacterial pathogens in hatcheries and nurseries must include routine sanitation of system surfaces, water filtration, brood stock sanitation and maintenance of low dissolved organic levels. Antibiotics have been used in experimental settings but are not routinely used on production scale systems due to cost as well as risk of producing resistant strains. A program to select and test probiotic strains of bacteria, as an alternative to antibiotic use, is underway and results to date will be presented.

Bacterial pathogens were first screened by comparing whole cell fatty acid profiles. Based on this evaluation, most pathogens were consistent or close to the Vibrio genus but probiotic candidates represented a variety of bacterial genera. Selected representative isolates were further characterized using biochemical criteria and 16s rDNA sequencing.

Candidate probiotic bacteria are first tested in agar plate inhibition tests. Strains showing inhibition to isolated pathogens are tested for haemolytic activity and pathogenicity to shellfish seed. Candidates passing these tests are then tested for inhibition of mortality and morbidity response in laboratory pathogen challenges.

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DEVELOPMENT OF AN ORAL ADMINISTRATION OF OXYTETRACYCLINE TO CONTROL LOSSES DUE TO WITHERING SYNDROME IN CULTURED RED ABALONE, *HALIOTIS RUFESCENS*

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Withering syndrome (WS) is a chronic and fatal disease of wild and cultured abalone, *Haliotis* spp. causing significant losses to commercial farmers and wild populations. The transmissibility of WS was tested by cohabitation of black abalone with WS and those without WS. Control abalone (+WS) died at a steady rate from week 1-41 with 92% cumulative mortality, while controls without WS experienced 12.5% mortality due to handling stress. Clinical signs of WS (mantle retraction and pedal muscle atrophy) were first observed ~21 weeks after initiation of the study in the experimentally infected animals followed by mortalities from week 36-46. Like animals exposed in the field, mortality of experimentally infected animals was rapid and high (79%). Microscopic examinations revealed numerous Rickettsiales- like procaryotes (RLPs) in digestive tissues accompanied by an atrophy and degeneration of the digestive gland only in abalone with WS. We observed an inverse correlation between RLPs and survival ($p<0.01$), and between RLP and condition of the digestive gland ($P<0.01$) and foot ($P<0.01$). These data suggest a role of the RLPs in WS. We subsequently determined that intramuscular injections of oxytetracycline were effective in treating the RLP infections and reducing or preventing associated losses. The causative agent of WS is an obligate intracellular bacterium (RLP), recently described as "*Candidatus* Xenohaliotis californiensis". In order to control losses due to this disease in abalone culture facilities, we are in the process of developing an oral administration of oxytetracycline. WS-positive, red abalone (~90g) were placed (n= 147each) into tanks (6 control and 6 treatments) and held at ~14-16℃. Experimental animals were fed a diet containing oxytetracycline for two weeks, while control animals were fed kelp. After the treatment, an El Nino event was simulated by turning off the flow of seawater to the 12 tanks during the day to produce an elevation of water temperatures from ~15℃ to ~18℃. After two months of daily temperature fluctuations, the animals were returned to ambient conditions. Two weeks after the final feeding of the medicated treatment, five abalone were sampled from each tank for histology and drug residue analysis. In addition, moribund animals from various tanks were sampled after 3 months. To date, we have observed significant reductions in the intensity of bacterial infections in both the postesophagus and digestive gland two weeks and four months after a two week treatment ($p<0.05$ and $p<0.001$, respectively). Losses of treated abalone (6.45%) were also significantly less than those in the unmedicated, control treatment (36.30%; $p<0.0001$). In addition, the biomass of treated animals exceeded (16.55kg) those of control animals (11.14kg, a 48% increase in total biomass) 10.6 months after a single treatment. All trials were conducted under the guidance of the FDA/CVM though their Aquaculture Investigational New Animal Drug program (INAD # 9332). We are presently beginning larger scale trials and establishing residue data for use in determination of a withdrawal time for this treatment regime.

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MOLECULAR APPROACHES TO UNDERSTANDING AND DIAGNOSING DISEASE IN MARINE INVERTEBRATES : DISEASE RESISTANCE, PATHOGEN ADAPTATIONS AND MOLECULAR PROBES FOR PARASITIC PROTISTA (*PERKINSUS* SPP.) AND TOXIC DINOFLAGELLATES (*PFIESTERIA* SPP.)

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Viral, bacterial, fungal and protozoan epizootic diseases are recognized as significant factors detrimental to the marine ecosystem, and the successful exploitation of natural and cultivated stocks of marine shellfish from coastal areas around the world. Currently, established methods for the control of disease in vertebrates, such as rational vaccination programs, can not be applied to invertebrate species. Further, the basic problem when considering enhancing disease resistance in marine invertebrates through transgenic approaches, is the identification of the suitable target gene (s). Specific and sensitive diagnostic molecular tools for early detection of pathogens in the host and the environment will aid in controlling disease through appropriate management strategies. In order to address the immediate need for a reliable diagnostic assay we developed a polymerase chain reaction (PCR) -based assay for the diagnosis of *Perkunsus marinus*, a protistan parasite of the eastern oyster, in oyster tissues selecting an intergenic, non-transcribed spacer (NTS) between the 5S and small subunit ribosomal RNA (SSU rRNA) genes as the target nucleotide sequence. The PCR-based assay is species-specific and can detect a single *P. marinus* trophozoite in 30 mg of oyster tissue, indicating that this diagnostic methodology is not only faster, but also more reliable than the current FTM assay. We implemented a similar approach to develop a sensitive and specific PCR-based assay for the detection of *P. piscicida*, a heterotrophic dinoflagellate that has been associated to massive fish mortalities and health problems in watermen working along the lower Eastern Shore of the Chesapeake Bay, and presumed to affect larval and juvenile shellfish. Based on genetic information, the PCR assay uses primers designed on the *P. piscicida* NTS and SSU regions, yielding an amplicon of 429 bp. We have been routinely applying this assay for the identification of *P. piscicida* in environmental water and sediment samples. Although it is a very sensitive technique for the detection of specific sequences in complex DNA mixtures, the PCR amplification can be inhibited by substances present in soil and sediments. In order to verify the amplification of the target sequence in sediment samples, we developed an internal standard that uses the same priming sites selected for the PCR-based assay for *P. piscicida*, but yields an amplicon of different size. PCR co-amplification of this internal standard in serial dilutions of sediment samples confirmed the presence of inhibitors of Taq polymerase, and revealed the lowest dilution at which the PCR amplification takes place. Thus, the inclusion of internal standards is critical for avoiding false negative results in the routine detection of *P. piscicida* in environmental samples [Supported by grants NOAA NA46RG0091, NIEHS 1PO1 ES09563; and ECOHAB NA860P0492]

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