Diseases in Asian Aquaculture VII

Editors

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Dr. Brian Jones
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Message from the President, Asian Fisheries Society

Congratulations to the AFS Fish Health Section for coming out with this Proceedings of its 7th Symposium on Diseases in Asian Aquaculture. The Society is always proud of your accomplishments, one of which is best demonstrated in your consistent ability to bring together, on an international scale, your members and colleagues to the regular triennial Symposium that has marked your dynamic existence since 1989. This seventh symposium held in Taipei, Taiwan on 22-26 June 2008 congregated over 300 participants representing 31 countries. The AFS council members were among those who witnessed some of the sessions, as a Council meeting was held immediately prior to your event also in Taipei.

Your theme "Communication, Cooperation and Coordination: Key Issues in Aquatic Animal Health Management" recognizes and promotes a new level of engagement among international biologists, pathologists, breeders, fisheries scientists and policy makers. It emphasizes not only the innovations and pioneering edge fostered by these scientists in aquatic disease, but also the responsibility to interact, exchange and share these intellectual and technological tools and products. Asia has the greatest drive to continually develop new technology and regimen to manage fish health because it is the leading aquaculture producing region in the world. By sharing Asia's scientific advances as well as problems through symposia such as this, synergy is created among scientists and other stakeholders from different countries, to enable them to collectively make a difference in addressing the constant challenges in the aquaculture industry.

The Proceedings will be a valuable resource that captures and preserves the knowledge shared during the Seventh DAA Symposium and will extend its reach to those who were not there. Furthermore, this publication of the Fish Health Section enhances the AFS as an organization and provides a model for other sections and branches. On behalf of AFS and its governing Council, I express our congratulations for your achievement and our wishes for your continued success!

Prof. Ida M.L. Siason, Ph.D.
President
Asian Fisheries Society (2009-2011)
November 2011

Message from the Chairperson, Fish Health Section of the AFS

The Seventh Symposium on Diseases in Asian Aquaculture (DAA VII) was held in Taipei City, Taiwan from June 22nd until June 26th 2008. The theme of the event was "Communication, Cooperation and Coordination: Key Issues in Aquatic Animal Health Management".

The Symposium housed more than 400 delegates from 31countries including: Afghanistan, Australia, Bangladesh, Brunei, Canada, China, Egypt, France, French Polynesia, India, Indonesia, Iran, Israel, Japan, Korea, Malaysia, the Netherlands, Norway, the Philippines, Singapore, Span, Sri Lanka, Taiwan, Thailand, the United Kingdom, the United States, and Vietnam to name a few. With this consortium of biologist, fishery scientist, breeders, veterinarians and the like, we were able to embrace the theme of "Communication and Cooperation" by exchanging ideas related to the business of aquaculture.

The Symposium topics:

- State of the world aquaculture
- Global perspectives in managing aquatic animal health
- Emerging issues in aquatic animal health management
- Epidemiology, detection and diagnosis of pathogens in fish, shellfish, mollusks and their environment
- Biosecurity and containment in aquaculture systems
- Recent developments in genomics, proteomics and bioinformatics: implications for aquatic animal diseases
- Immunology/Disease resistance/Host-pathogen interaction
- Microbiology of aquatic animal pathogens and antimicrobial peptides
- Disease of aquatic invertebrates -Shrimp health
- Disease of aquatic invertebrates -Mollusks health
- Disease of aquatic vertebrates -Finfish health
- Pathogen risk analysis, probiotics, therapeutics and other fields
- The way forward
  - o Aquatic animal health management
  - o International trade, aquatic animals and risk were fully developed within the context of 16 keynote speeches, 11 lectures, 56 oral presentations and some 204 posters.

The highlight of the Symposium was the publication of *Diseases in Asian Aquaculture VII*. Via the publication of this work, we hope that its contents will provide direction, insight and new knowledge to advance the quest for better aquatic health management in Asian aquaculture.

On whole the interdisciplinary discussions, shared research, contributions of our sponsors and efforts put forth by the organizers, combined with the closing keynote speeches by Dr. Barry Hill ad Dr. Timothy Flegel were invaluable to supporting the theme of "Communication, Cooperation and Coordination."

Having highlighted the successes of the proceedings, I would like to thank the Asian Fisheries Society, The Fisheries Society of Taiwan, Academia Sinica, The Fish Health Section, the College of Life Sciences, NTU, Uni-President, PHARMAQ, the delegates, the speakers and each and every person that had a hand in making the Seventh Symposium on Diseases in Asian Aquaculture (DAA VII) a success. In addition, I extend my appreciation to the editorial board, Dr. Melba B. Reantaso (chief editor), Dr. Brian Jones, Dr. Flavio Corsin and Prof Takashi Aoki, for their contributions.

Prof. Chu Fang Lo
Chairperson
FHS-AFS Executive Committee (2008-20011)

Message from the Editors, DAA VII

The disease situation in aquaculture is changing rapidly in unpredictable ways due to the current period of rapid change in the international trading environment and globalization. Live animals are moved from place to place, country to country and region to region as broodstock, postlarvae, fry and fingerlings. This expanded and occasionally irresponsible global movements of live aquatic animals have been accompanied by the transboundary spread of a wide variety of disease agents that have caused serious damage to aquatic food productivity and resulted in serious pathogens becoming endemic in culture systems and the natural aquatic environment.

The Asia-Pacific, the dominant aquaculture region in the world has been facing serious threats from aquatic disease emergencies during the last few years. Old and emerging transboundary aquatic animal diseases have continued to challenge the sustainability of the sector. In addition, the use of specific pathogen free (SPF) stocks without appropriate biosecurity support resulted in the introduction of new pathogens exotic to the region.

The Fish Health Section continues to play its important role as a major networking platform and source of information through original research publications and other preventative health management strategies and through the Diseases in Asian Aquaculture (DAA) symposia, where interaction with fellow researchers/scientists and dissemination and exchange of research and project-related findings on aquatic animal health take place.

We are pleased to present this seventh volume of the DAA series which brings together original research and review papers that were presented during the Seventh Symposium on Diseases in Asian Aquaculture (DAA VII) held in Taipei, Taiwan from 22-26 June 2008. A total of 45 papers, submitted for inclusion in the proceeding, were sent out to international expert-reviewers. After a robust review process, 26 papers were finally accepted forming the contents of this publication.

A voluntary work and a daunting task, we are pleased to be part of this primary publication of our society. We appreciate the opportunity to contribute to this well-established, standardized series (to-date 7 volumes spanning close to the 24-year history of the FHS) that is well known, easily recognized by its distinctive blue and gold volumes, well cited and held in high opinion by fish health workers throughout the world.

We sincerely thank the international expert-reviewers who generously assisted scientists from less developed countries to bring the scientific quality and English usage of their manuscripts up to international standards. We would also like to apologise to all the contributors for any inconvenience resulting from the tedious process which we had to endure.

Special thanks also goes to Dr. Chu Fang Lo, Dr C.V. Mohan and the members of the FHS Executive Committee for their relentless support and Ms Marika Panzironi for various types of assistance to complete this volume.

We expect that free downloadable copies of all articles will be available at the same time from the FHS website, in support of the ‘open archive movement’ intended to make science readily available to all, especially for publicly funded research.

Getting the DAA series published on time is becoming more and more difficult because of the voluntary nature of the service and because of the lack of a mechanism to efficiently go though the entire publication cycle. We still do not know what will be the fate of the DAA series, but we earnestly hope that members and supporters of FHS will continue to dedicate a portion of their work to this endeavour to assist our society and in support of the FHS long standing tradition of producing DAA proceedings.

Melba G. Bondad-Reantaso
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Diseases of finfish

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Significant and emerging parasitic diseases of finfish

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ABSTRACT

Fish parasites constitute widely diversified groups of unicellular and multicellular organisms. Recently, less known parasite groups have emerged as serious pathogens. Firstly, infection with dinoflagellates of the genus *Ichthyodinium* has been found during seed production of leopard coral grouper, *Plectoropomus leopardus*, in Japan and yellowfin tuna, *Thunnus albacares*, in Indonesia. Schizonts multiply in host ova, leading to burst of yolk sacs of embryos or hatched larvae. The complete life cycle is unknown, but there is evidence that the infective stage was present in the rearing water. Secondly, heavy infections of endoparasitic turbellarians have been noticed in hatchery-reared stonefish, *Inimicus japonicus*, fry and cage-cultured sea perch, *Lateolabrax* sp. and greater amberjack, *Seriola dumerili*. Furthermore, wild Japanese sea perch, *Lateolabrax japonicus*, and cultured red sea bream, *Pagrus major*, were concurrently infected. It is not clear whether these turbellarians, probably belonging to the genus *Paravortex*, comprise a single species. Hitherto unknown parasitic diseases among wild fish should also be noted: the monogenean, *Neoheterobothrium hirame*, has been a serious threat to wild olive flounder, *Paralichthys olivaceus*, since its sudden appearance in the early 1990s. Circumstantial evidence and molecular data strongly suggest its natural host is southern flounder, *Paralichthys lethostigma*, in North America, and that infected fish was introduced into Japanese waters. Among freshwater fishes, metacercariae of the bucephalid trematodes, *Parabucephalopsis parasiluri* and *Bucephalopsis ozakii*, and the leech, *Limnotrachelobdella sinensis*, have been found in wild cyprinid fishes of Japan. It is suspected that these parasites were introduced through importation of live shellfish and fish, respectively. These cases in the wild are more serious than those occurring in aquaculture, as it is unrealistic to take control measures for the containment of diseases of wild fish.

Key words: emerging disease, parasite, *Ichthyodinium*, *Paravortex*, *Neoheterobothrium hirame*, *Parabucephalopsis parasiluri*, *Limnotrachelobdella sinensis*

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INTRODUCTION

With increasing demands for specific pathogen-free seeds in aquaculture, more and more species of culture seeds have recently been produced in an ever larger scale than before. This practice is accompanied by frequent outbreaks of infectious diseases in hatcheries. While most diseases are caused by viral agents, protozoan and metazoan infections do occur in hatcheries, but have not yet been sufficiently documented.

In contrast to aquaculture, diseases of wild fish and shellfish have been recorded much less frequently. This is mainly because diseased animals are easy prey to predators before we notice their presence. Diseases caused by parasites are no exceptions, and only a few cases have so far been documented in wild populations (Jones, 2005). Although much attention is focused on the economic loss due to parasitic diseases in aquaculture, impacts of the parasitic infections on wild populations must not be neglected.

In many cases, importation of foreign fish and shellfish has been deeply involved in the emergence of new diseases among indigenous species. In Japan, legislation was introduced in 1996 to prevent foreign fish and shellfish pathogens from entering into Japanese waters. Under the law for conservation of aquatic resources, we request the exporting countries to issue specific pathogen-free certificates for selected diseases of selected aquatic animals. At present, they are Spring Viremia of Carp (SVC) and Koi herpesvirus (KHV) disease for carp *Cyprinus carpio* (common carp and koi carp), SVC for some cyprinid fish including goldfish *Carassius auratus*, Viral Hemorrhagic Septicemia, Epizootic Hematopoietic Necrosis, Piscirickettsiosis and Enteric Redmouth Disease for salmonid eyed-eggs and juveniles, Nuclear Polyhedrosis Baculoviroses, Yellow Head Disease, Infectious Hypodermal and Hematopoietic Necrosis and Taura Syndrome for penaeid shrimp juveniles. Further, once introduced, we try to contain these specified diseases under the law for sustainable aquaculture production. For KHV, since the introduction of the virus in 2003, efforts have been directed to the prevention of the virus from spreading to still uncontaminated areas in Japan. In other words, we do not have any legislation to stop importation of foreign aquatic animals harbouring other pathogens than the listed ones above, as long as they are apparently healthy upon importation.

In this short review, I would like to present examples of lesser known parasitic diseases in the wild and in hatcheries, which I recently encountered. It is almost certain that newly emerging diseases in wild fish described here are all caused by pathogens introduced from abroad. Diseases in hatcheries occur in close association with those in the wild and possibly also in growout facilities. Thus, for the establishment of effective control measures in hatcheries, it will be important to demonstrate the life cycle and to investigate the entire infection cycle among hatcheries, growout facilities and wild environments.

PARASITIC DISEASES IN SEED PRODUCTION FACILITIES

Infection of fertilized eggs by the dinoflagellate, *Ichthyodinium chaberardi*, has been known to occur among marine fish including Atlantic sardine, *Sardina pilchardus*, and similar infections have been described in the eggs of Atlantic cod, *Gadus morhua*, and turbot, *Scophthalmus maximus*, in hatcheries (Noga and Levy, 2006). These occurrences have so far been limited in Europe, but, since 2000, very similar infections have been occurring in the seed production of leopard coral grouper, *Plectropomus leopardus*, on Ishigaki Island, southwestern Japan (Mori *et al*., 2007) and of yellowfin tuna, *Thunnus albacares*, in Indonesia (Yuasa *et al*., 2007). There were considerable fluctuations in the annual occurrence, but once it occurred, high mortality was induced. Schizonts of the parasite multiplied in the yolk sac of fertilized eggs (Fig. 1) or hatched larvae (Fig. 2) and parasite-filled yolk sac eventually burst. The parasite released into the water transformed into the dinoflagellate stage, the fate of which is unknown. Although the infective stage has not been specified, there is evidence that their life cycle is established among local wild fish populations, and fertilized eggs of the grouper in hatcheries got infected through contaminated water from outside. This infection was effectively controlled by treating the rearing water with ozone at 0.5 mg/l for more than one minute (Table 1) (Mori *et al*., 2007).

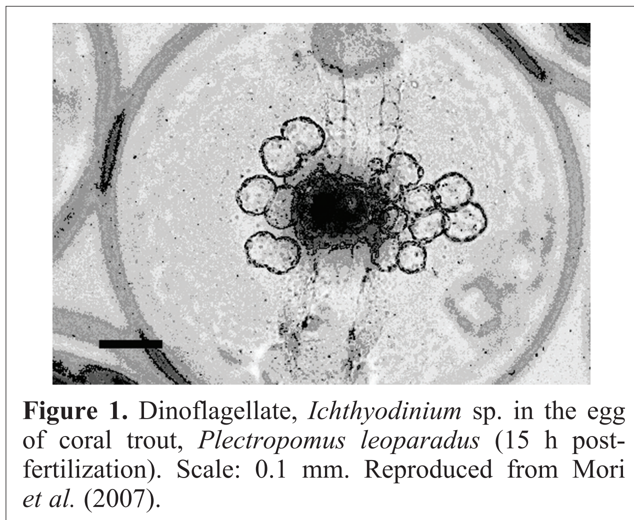

Figure 1. Dinoflagellate, *Ichthyodinium* sp. in the egg of coral trout, *Plectropomus leoparadus* (15 h post-fertilization). Scale: 0.1 mm. Reproduced from Mori *et al.* (2007).

Some groups of turbellarians are fish parasites, while the majority are free-living or commensal with other animals. Among them, *Paravortex* sp. (possibly *Paravortex* spp.) infects many wild fish species (Cannon and Lester, 1988), and, in captivity, caused mortality among aquarium fish (Kent and Olson, 1986). This parasite is viviparous (Fig. 3), and, when mature, leaves host to give birth to its offspring in the water.

In Japan, *Paravortex* infection is a serious threat in the seed production of a stonefish, *Inimicus japonicus* (Family Synanceiidae). The parasite is endoparasitic, invading the gills, skin and fins of fry. The source of infection is thought to be spawner stonefish, which

Table 1
Experimental control of Ichthyodinium infection of fertilised eggs of Plectropomus leopardus by sterilisation of seawater used to rear broodstock (data based on Mori *et al.* (2007).

Experimental group	**Occurrence (%) (Infected egg batches/total egg batches)**	
	2003	**2004**
Spawning in disinfected seawater (Treated with oxidant)	0% (0/82)	0% (0/136)
Spawning in untreated seawater	7.30% (6/82)	10.30% (7/68)

got infected in the wild. This may have been the result of fry being reared in sea water contaminated with young parasites released from adult parasites. This turbellarian was also found in wild rockfish, *Sebastiscus marmoratus* (Ogawa, unpublished data) in 1980s and wild Japanese sea perch, *Lateolabrax japonicus*, in 2006 (N. Akao, pers. comm.). *Paravortex* infection has also recently been noticed among cage-cultured fish in 2006: amberjack, *Seriola dumerli*, imported from China, Chinese sea perch, *Lateolabrax* sp., red sea bream, *Pagrus major*, and the stonefish, *I. japonicus*. Fecundity of the parasite is still largely

Figure 2. Coral trout, *Plectropomus leoparadus* immediately after hatch with full of schizont stage dinoflagellates. Scale: 0.5 mm. Reproduced from Mori *et al.* (2007).

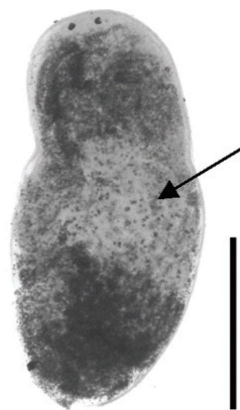

Figure 3. *Paraveotex* sp. infecting stonefish, *Inimicus japonicus*. Arrow indicates larvae in the uterus. Heidenhain's iron hematoxylin stain. Scale: 0.5 mm.

unknown except that the number of offspring an adult produced each time ranged from 62 to 248 (mean: 133) (Fukuda, Y., Miyoshi, Y. and Ogawa, K. 2006). It caused mortality among *I. japonicus* fry reared in hatcheries and in culture tanks. Chemotherapy seems ineffective because of its endoparasitism, and prevention of infection by using parasite-free water will be a realistic control method in the hatchery.

PARASITIC DISEASES OF WILD FISHES

A new diclidophorid monogenean, *Neoheterobothrium hirame*, was found on the gills and buccal cavity wall of wild olive flounder, *Paralichthys olivaceus*, collected from the Sea of Japan (Ogawa, 1999) (Fig. 4). Infected fish sometimes show severe anaemia caused by the blood feeding of the parasite (Yoshinaga *et al*., 2000). Infection was first noticed among wild olive flounder in 1995, but subsequent examination of flounder specimens collected in 1989-1999 and preserved in formalin (n=841; 0- and 1- year old) in Niigata Prefecture on the Sea of Japan side confirmed the infection of fish collected in 1993 and later, but not from fish in 1989-1992 (Anshary *et al*., 2001).

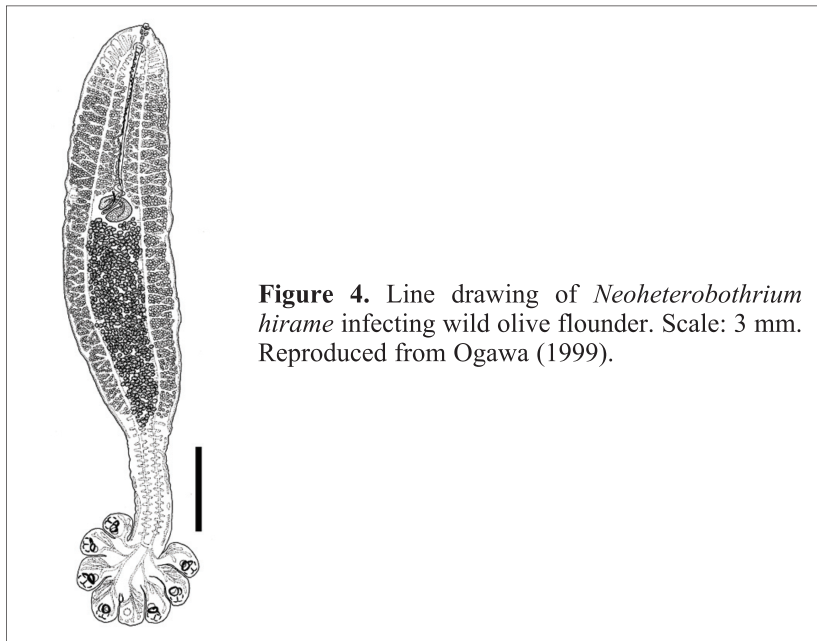

Figure 4. Line drawing of *Neoheterobothrium hirame* infecting wild olive flounder. Scale: 3 mm. Reproduced from Ogawa (1999).

This strongly suggests that the monogenean appeared suddenly in Japanese waters in early 1990s. Since the first confirmation of infection in 1993, *N. hirame* was found in wild flounder from Hokkaido to Kyushu to cover the whole area of the host distribution in six years (Fig. 5). This rapid expansion of the parasite's geographical distribution was probably due not only to host migration, but also to human activities by transporting flounder between the Sea of Japan side and the Pacific side. Flounder of the year are quite susceptible to infection, and the catch has drastically declined especially in western Japan (Anshary

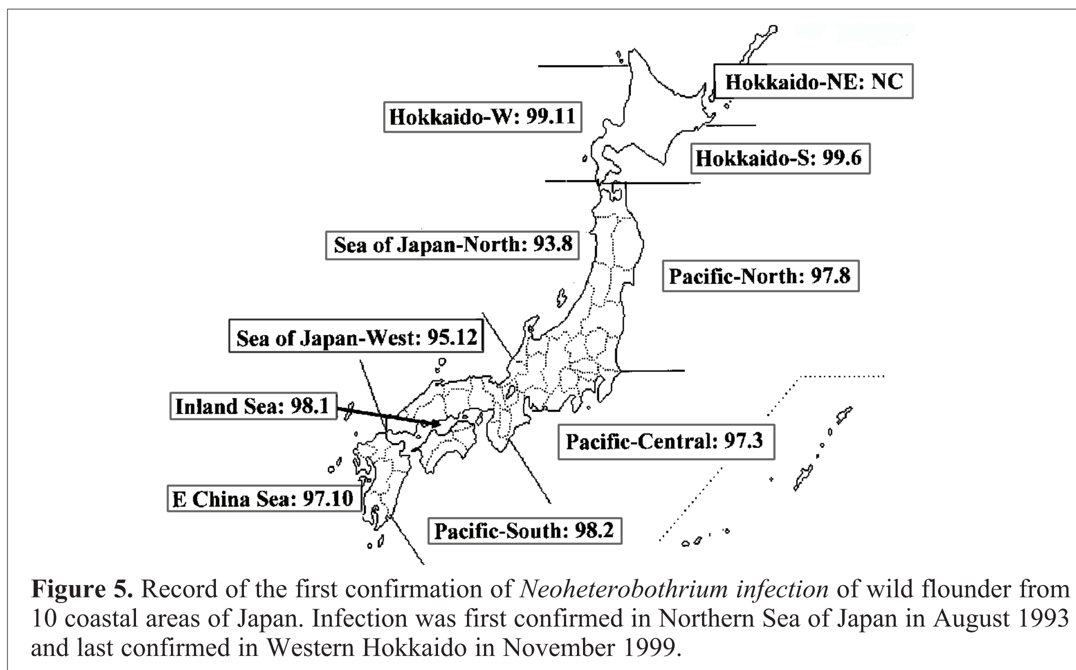

Figure 5. Record of the first confirmation of *Neoheterobothrium infection* of wild flounder from 10 coastal areas of Japan. Infection was first confirmed in Northern Sea of Japan in August 1993 and last confirmed in Western Hokkaido in November 1999.

et al., 2002). Infection experiments suggest that *N. hirame* induces behavioural changes in infected flounder, making them vulnerable to predation by larger flounder (Shirakashi *et al*., 2008). This may be responsible for the recent decline in the flounder populations of Japan.

The Yodo River system originates in Lake Biwa, passing through two dams to lead into the Uji River and then, the Yodo River, finally flowing into Osaka Bay. Since 2000, hitherto unknown parasites have appeared almost coincidentally: metacercariae of bucephalid trematodes, *Parabucephalopsis parasiluri* and *Bucephalopsis ozakii*, were found encysted in many cyprinid fish, most notably in *Zacco platypus* and *Squalidus chankaensis* in the Uji River and Yodo River (Ogawa, K., Nakatsugawa, T. and Yasuzaki, M. 2004; Urabe *et al*., 2007) (Fig. 6), and a leech, *Limnotrachelobdella sinensis*, infecting crucian carps, *Carassius cuvieri* and *Carassius langsdorfii*, in the Yodo River (Ogawa, K., Rusinek, O. and Tanaka, M. 2007) (Fig. 7). Heavy metacercarial infection in the eye and fins caused hemorrhages. These trematodes involve a freshwater bivalve, *Limnoperna fortunei*, as the first intermediate host with fish, mostly cyprinids, as the second intermediate hosts and Lake Biwa catfish, *Silurus biwaensis*, and Amur catfish, *Silurus asotus*, as the final hosts (Ogawa, K., Nakatsugawa, T. and Yasuzaki, M. 2004; Urabe *et al*., 2007). It is probable that the trematode was introduced from eastern Asia to Japan through infected bivalve, *L. fortunei*. With the presence of potential second and final hosts in this river system, the life cycle of these bucephalid trematodes started to be completed there. Since the first confirmation, the distribution of the parasite has been restricted to the Uji River-Yodo River area, but very recently in 2007, infected bivalves were found in the upstream of the two dams between

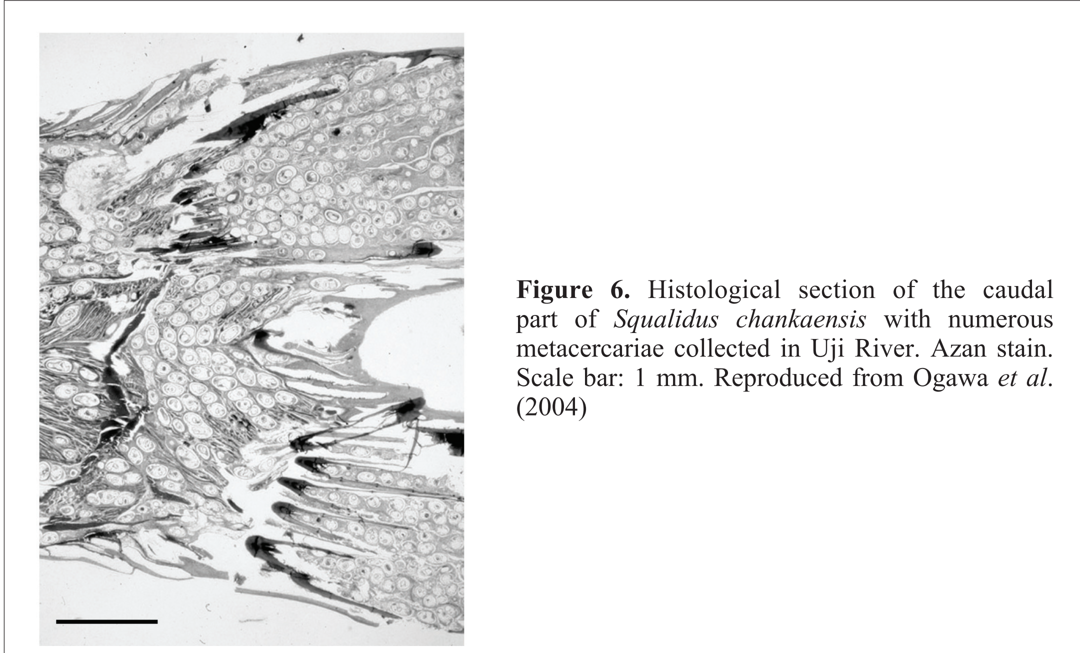

Figure 6. Histological section of the caudal part of *Squalidus chankaensis* with numerous metacercariae collected in Uji River. Azan stain. Scale bar: 1 mm. Reproduced from Ogawa *et al*. (2004)

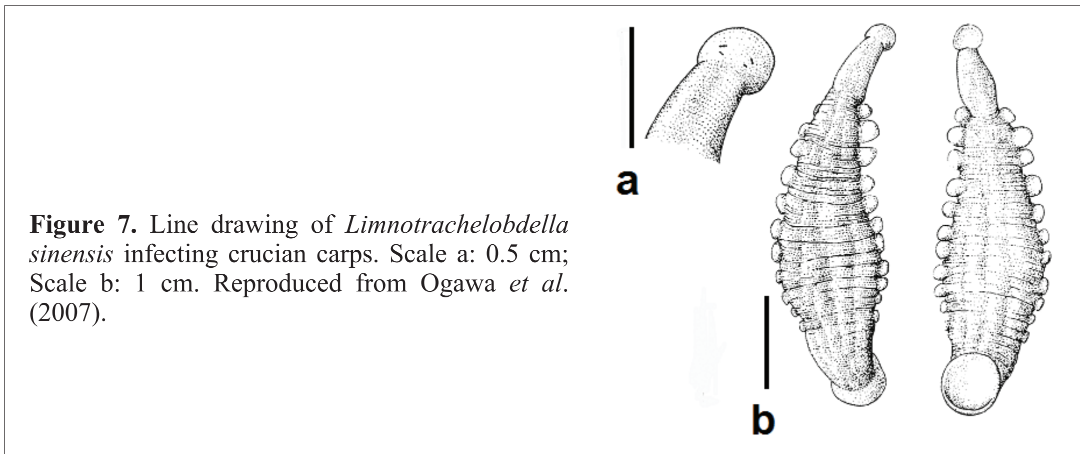

Figure 7. Line drawing of *Limnotrachelobdella sinensis* infecting crucian carps. Scale a: 0.5 cm; Scale b: 1 cm. Reproduced from Ogawa *et al*. (2007).

Lake Biwa and the Uji River (M. Urabe, personal communication) (Fig. 8). The parasite has expanded its distribution upstream beyond the Uji River possibly by accidental introduction of infected cyprinids or catfish. Now that the parasite is present just downstream of the Lake Biwa, where many unique indigenous cyprinid fish are inhabited, it will be a great risk to such fish, which may be as susceptible as the cyprinid fish in the Uji River.

The origin of the leech, *L. sinensis*, found in the Yodo River, is not clear since it suddenly appeared in 2000 (Ogawa, K., Rusinek, O. and Tanaka, M. 2007). It is quite unlikely that the parasite, up to 5 cm in body length, has long been overlooked, assuming that it *is* an indigenous species to Japan. It is rather natural to consider that the leech was introduced

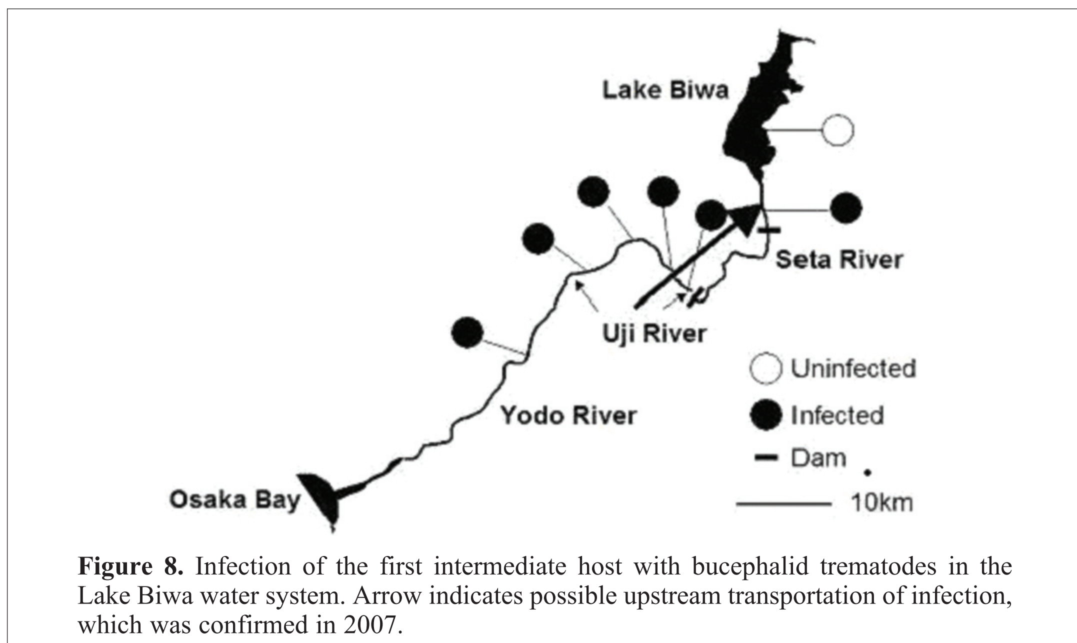

Figure 8. Infection of the first intermediate host with bucephalid trematodes in the Lake Biwa water system. Arrow indicates possible upstream transportation of infection, which was confirmed in 2007.

through infected crucian carp from eastern Asia, where it is known to infect common carp and crucian carps. The parasite appears to be pathogenic, since infected fish showed anemia. For the moment, there is no evidence that the leech has expanded its distribution beyond the Yodo River, but the host fish, *C. cuvieri* and *C. langsdorfi*, are widely distributed in Japanese waters, and once introduced, there is a risk that the parasite life cycle becomes established in a new locality.

Since control of infections of wild fish is not possible, care should be taken not to transport such potential hosts from these contaminated areas to naïve areas.

CONCLUDING REMARKS

It is almost certain that the causative parasites of the emerging diseases among wild fish populations described here were of foreign origin. In the case of *Neoheterobothrium hirame* infection of olive flounder, it is strongly suggested that the monogenean is originally the parasite of southern flounder, *Paralichthys lethostigma*. This means that the parasite has switched its host from an American flounder to the native Japanese flounder. Such host switch would never have happened without the human activity to introduce the foreign flounder from North America to the Far East, which was totally an unexpected event. It was also unexpected that the bivalve, *L. fortunei*, probably attaching to freshwater bivalves imported for food consumption (Urabe *et al*., 2001), was infected with trematodes new to Japan, which have established their life cycles in Japanese waters.

It should be noted that *N. hirame* has spread over the whole area where olive flounder is naturally distributed, in just six years. Similarly in the Yodo River system, the bucephalid trematodes (or at least one of the two species) have expanded their distribution upstream, posing a great threat to wild fish populations in the Lake Biwa, while the geographical distribution of the leech, *L. sinensis*, is still limited. It seems unlikely that olive flounder dispersed from the Sea of Japan side to the Pacific side in such a short period, and that cyprinids swam upstream from the Uji River across the two dams. It is more likely that human activities may have been involved in the expansion of these parasites' distribution.

Since control measures against diseases in wild fish populations are almost impossible and since we cannot anticipate the consequence of any host switch as in the case of *N. hirame* infection of olive flounder, it is clear that introduction of live foreign aquatic animals should be done responsibly and cautiously. Domestic production of culture seeds may prevent further introduction of foreign pathogens.

Knowledge about outbreaks of parasitic diseases in hatcheries and in the wild has been very limited, compared with those occurring in growout facilities. Two new parasitic diseases in hatcheries were described here, one by the dinoflagellate, *Ichthyodinium* sp., and the other by the turbellarian, *Paravortex* sp. (or *Paravortex* spp.). In both infections, the causative agents transmit horizontally, i.e. the source of infection was thought to be contaminated water containing the infectious agent in the *Ichthyodinium* infection and newly born offspring from matured turbellarians which had infected spawners in the *Paravortex* sp./spp. infection.

It is very important to understand the infection cycle of these parasites in the hatcheries. In the case of *Ichthyodinium* infection, there may be a persistent infection among wild fish, and rearing water pumped into the facilities is contaminated with the infectious agent. Thus, transmission can be prevented by disinfecting the rearing water. In the case of *Paravortex* infection, it is likely that the spawner had already been infected with the turbellarian and the infection cycle started to be completed within the culture facility involving not only the spawner but also its progeny. Since eradication of the parasite from spawners is very difficult, prevention measures should be taken by rearing fry using a different water source from that of the spawner.

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Recent developments in the study and surveillance of koi herpesvirus (KHV) in Asia

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ABSTRACT

Koi herpesvirus infection causes significant mortalities in common carp (*Cyprinus carpio carpio*), koi carp (*Cyprinus carpio koi)* and ghost carp *(common x koi cross, Cyprinus carpio koi*). Outbreaks have been reported in many countries worldwide i.e. UK, Germany, Israel, USA, Belgium, South Africa, Switzerland, The Netherlands, France, Denmark, Austria, Italy, Luxemburg and Poland. The first outbreaks attributed to KHV in Asian countries were reported from Hong Kong in 2001; Indonesia in 2002; Taiwan in 2002; Japan in 2003; Thailand in 2005; and Singapore in 2005. Thereafter, research studies embarked on KHV focused on pathogenicity, cell line susceptibility, fish size susceptibility, predilection to fish organs, persistence in fish, vaccine development and application, surveillance and gene sequence analyses of KHV strains. To date, annual active surveillance of the virus in Cambodia, Lao PDR, Myanmar, the Philippines and Vietnam showed that these countries were free of KHV from 2004 to 2007. Several strains of KHV apparently affect koi and common carp in this region indicating that transboundary movement of the virus has occurred not only in Asia but also from Europe and the Americas. The extensive international trade in live ornamental koi fish has largely contributed to the global spread of KHV. Hence, KHV disease (KHVD) was recently added to the list of notifiable diseases of the World Organisation of Animal Health or the Office International des Epizooties (OIE), an indication of the global significance of this viral infection.

Key words: koi herpesvirus (KHV), *Cyprinid herpesvirus* 3 (CyHV-3); outbreaks, Asia, genotypes, pathogenicity, surveillance, vaccine

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INTRODUCTION

The common carp (*Cyprinus carpio carpio*) is the third most important farmed freshwater fish species in the world (FAO Yearbook, 2004). The main carp-producing countries are found in Europe, Asia, Russia, South America and parts of Africa. In many Asian countries, common carp are cultured widely as foodfish. In Indonesia, for instance, carp is the national fish species of the country. The ornamental variety, koi carp (*C. carpio koi*), is also popular worldwide, as pets. In fact, choice koi carp, which are very expensive, are bred in Japan, Israel and some Southeast Asian countries for ornamental fish hobbyists. As such, international trade in koi carp has provided routes for transboundary introduction of koi herpesvirus (KHV), also known as *Cyprinid herpesvirus* 3 (CyHV-3), into naïve populations in many countries and more recently in Southeast Asia (Haenen and Hedrick, 2006; Lio-Po, 2007).

Koi herpes virus infection causes significant mortalities in koi and common carp, *C. carpio*. The disease was earlier known as carp interstitial nephritis and gill necrosis (CNGV) in Israel (Ronen *et al*., 2003; Pikarsky *et al.,* 2004). The first outbreaks of KHV were reported in Israel and Germany in 1998. Subsequent outbreaks were reported in USA, England and The Netherlands. To date, the disease has been reported in Belgium, South Africa, Switzerland, France, Denmark, Austria, Italy, Japan, Luxemburg and Poland (Haenen *et al*., 2004; Bergmann *et al*., 2006). Interestingly, polymerase chain reaction (PCR) testing of archived samples of an earlier unexplained mass mortality of koi and common carp in the UK confirmed that KHV was already present there as early as 1996 (Denham, 2003).

GEOGRAPHIC OCCURRENCES IN ASIA

In Asia, the first outbreak of KHV that caused mass mortalities of cultured common carp and koi carp occurred in Israel in the spring of 1998 when water temperatures were 18-28°C. By the end of 2001, 90% of carp farms in Israel reported disease outbreaks attributed to KHV (Perelberg *et al.,* 2003). Other first reports of disease attributed to KHV occurred among koi in Hongkong in 2001; common carp in Indonesia, in 2002; koi in Taiwan, in 2002; common carp in Japan, in 2003; koi in Thailand and Singapore, in 2005 (Haenen and Hedrick, 2006; Lio-Po, 2007).

The initial case of KHV mortality in Indonesia occurred among koi carp cultured in Blitar, East Java, in March 2002. The site had a history of fish introduction with fish imported from China through Hong Kong in January 2002 (Sunarto *et al.*, 2005a). The following month, a second disease outbreak occurred among cultured common carp in Subang Regency, West Java. The infected fish manifested clinical signs similar to the infected koi in Blitar. By May and June of the same year, common carp cultured in floating net cages in Cirata Reservoir, West Java, were also hit by the virus. The following year, outbreaks were reported in several carp farms in other parts of the country and by 2006 the disease had spread to many more sites (Fig. 1). The outbreak was associated with 95% mortality and losses amounting to approximately US$0.5 million within a 3 month period (Sunarto *et al.*, 2005b).

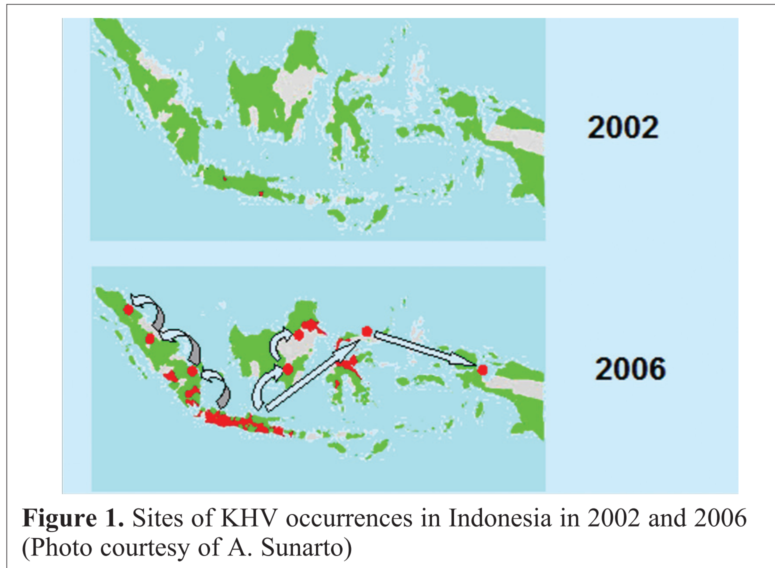

Figure 1. Sites of KHV occurrences in Indonesia in 2002 and 2006 (Photo courtesy of A. Sunarto)

At about the same time, the first occurrence of KHV infection emerged in two 2-year old koi carp in a private pond in Taipei County, Taiwan in December 2002 (Tu *et al.*, 2004a). During the following year, 3 more separate cases of 2-3 year old koi carp with populations of 20 (pond), 300 (artificial lake) and 700 (lake) occurred in Taipei, Taiwan and in 2004, another KHV outbreak occurred in a private koi hatchery in Taipei County, Taiwan (Tu *et al.*, 2004b).

In Japan, KHV epizooties was first reported among cage-cultured common carp in Lake Kasumigaura, Ibaraki Prefecture in October 2003 when water temperature in the lake was 16-18°C (Sano *et al.*, 2004a). By mid-November, the virus caused mass mortality of approximately 1,200 metric tonnes of common carp cultured in the lake. Archived samples from an earlier case of >10,000 common carp mortalities in May to July 2003 in some rivers and a lake in Okayama Prefecture were subsequently confirmed as KHV outbreaks. By the end of that year, KHV was detected in 23 of 47 prefectures. From January to May 2004, KHV was detected in 24 of 47 prefectures (Sano *et al.*, 2004b). Moreover, mass mortality of carp attributed to KHV occurred in seven rivers in Kanagawa Prefecture in May/June 2004 (Hara *et al.*, 2006). By 2005, 42 of 47 prefectures had confirmed cases of the virus. By spring 2006, KHV was detected in 45 of 47 prefectures and involved koi carp for the first time (T. Iida, *pers. commun.*). Such dramatic spread of the disease in Indonesia and Japan created an acute awareness of its emergence as a new disease in the region with predictably alarming implications.

In Thailand, KHV was first diagnosed in koi carp of a fish hobbyist in Bangkok in 16 March 2005 (Tandavanitj *et al.*, 2005). It was noted that only a few days earlier, the fish had participated in a koi competition held in Bangkok. After the contest, some of the koi

developed clinical signs and were confirmed as KHV-positive by PCR. Fortunately, the disease was limited to the koi companies that joined the competition and was reportedly contained. Mortalities ranged from 5 to 30%.

In Singapore, KHV was detected in two batches of Thai koi carp exported to Singapore, in February 2006. The fish tested KHV-positive by PCR and the virus was successfully isolated on KF-1 cells (S. Kueh, *pers. commun.*). During the period April to June 2006, koi carp imported to Singapore from Malaysia also tested positive for KHV. Earlier, the KHV was also detected among koi in Malaysia by nested PCR in 2004 but was not associated with epizootic outbreaks (Musa *et al.*, 2005). Even earlier, in another example of exported koi, the presence of KHV was detected among koi carp exported to the United Kingdom from Malaysia in 2000 and in 2001 (Gilad *et al.*, 2003).

Surveillance of the virus was carried out in both common and koi carp of Cambodia, Lao PDR, Myanmar, the Philippines and Vietnam through the auspices of the Government of Japan and the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD). The three-year active surveillance study covered 1481 common and koi carp sampled during the periods December 2004 to February 2005, September 2005 to February 2006 and October 2006 to February 2007. KHV was not detected using either cell culture, bioassay or PCR in any of the fish samples (Lio-Po *et al*., 2009). Moreover, no related epizooties among common and koi carp were observed by the fish farmers.

CAUSATIVE AGENT, GENOTYPES AND STRAINS

This disease is attributed to infection by Koi herpesvirus (KHV) or *Cyprinid herpesvirus* 3 (CyHV-3) of the family, *Herpesviridae* (Fig. 2) (Waltzek *et al.*, 2005). This double-stranded DNA virus has been isolated by cell culture from infected stocks of koi or common carp in many countries including the United States of America (USA), the UK, Israel, Japan, Indonesia, Malaysia, Thailand and Taiwan. The approximate diameter of KHV virion is 100-110 nm (Ronen *et al.,* 2005). The total genome length of KHV is approximately 295 kbp (Aoki *et al.*, 2007). Sequence analyses determined that KHV isolates from UK, USA and Israel were very similar. In other studies, the genotype of the USA strain was classified as E1, the Israel KHV as E6 strain while the Netherlands strains consisted of E1, E2, E3, E4 and E5 genotypes (Kurita *et al.*, 2008). Among the Asian KHV isolates, the Japanese KHV was classified as strain A1, the Indonesian strains as A1 and A2, the Taiwanese strains as A1 and A2 while the PR China KHV as an A1 strain (Lio-Po *et al.*, 2006; Kurita *et al.* 2008). Thus, it appears that the KHV strains found in The Netherlands, USA and Israel are closely related while those moving about Asian countries are, likewise, closely related.

Variation in the virulence of two strains of KHV was experimentally demonstrated by Yuasa *et al.* (2007). Strain 1 caused 50% mortality while strain 2 caused 60-75% mortality in common carp. In addition, strain 1 was detected by PCR in the gills, scales, kidney, spleen, liver, heart, intestine and brain 14 days post-infection (dpi) and in the gills, scales, kidney 28 dpi while strain 2 was initially detected in the 8 organs much later at 28 dpi and in the

gills, scales, kidney, intestine and brain by 60 dpi. Interestingly, strain 2 was detected in the brain over a long time period (360 dpi). At 70 dpi, >10,000 genome copies/mg wt. of the brain compared with a mean of <1 000 genome copies/mg wt. of the kidneys of surviving fish were detected.

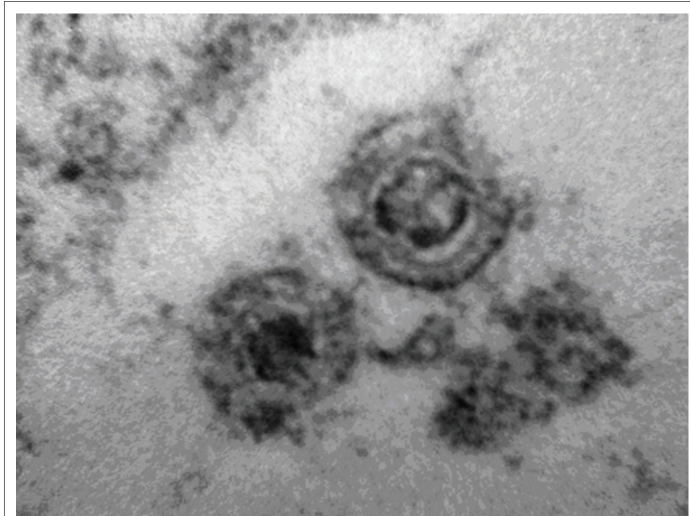

Figure 2. Transmission electron photomicrograph of KHV (Photo courtesy of K. Way).

FISH SPECIES AFFECTED

KHV infects common carp, koi carp and ghost carp (hybrid of koi and common carp) causing 30-100% mortality. Experimental transmission of KHV from common carp to goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) or tilapia (*Oreochromis niloticus*) failed to induce KHV infection (Perelberg *et al.*, 2003). However, Hedrick *et al.* (2006) reported that goldfish x common carp hybrids were moderately sensitive to KHV infection. Likewise, tilapia (*O. niloticus*) and catfish (*Pangasius hypophthalmus*) upon cohabitation with KHV-infected common carp did not develop disease nor was KHV detected by PCR (Yuasa and Sano, 2009. In Indonesia, the common carp naturally-infected with KHV were cultured in cages adjacent to caged tilapia, but the latter fish did not succumb to infection.

GROSS CLINICAL SIGNS

Infected fish typically manifest white, necrotic patches on the gill filaments (Fig. 3). Thus, affected fish often swim on the surface and exhibit respiratory distress. Other clinical signs may consist of sunken eyes, pale patches on the body surface, excessive mucus production and rough pale patches of the skin. However, some infected fish may show no visible signs of disease. Microscopic examination of the gills often shows associated bacteria and parasites (Hedrick *et al.*, 2000).

Figure 3. Koi carp with KHV showing varying severity of whitish or necrotic gills (Photo courtesy of A. Sunarto).

PATHOLOGY

KHV is very virulent; natural infection often causes 80-90% mass mortality of koi and common carp within 1 week (Perelberg *et al.,* 2003; Sano *et al.,* 2004a; Tu *et al.,* 2004a; Sunarto *et al.,* 2005b). Experimental infection using cell-cultured KHV induced 75-95% mortalities in koi and common carp (Pikarsky *et al*., 2004). The disease is very contagious for koi and common carp but not for humans. The disease has an incubation period of 5-7 days and is characterized by sudden onset, rapid spread when water temperatures are in the range 15-25°C (Gilad *et al*., 2003; Ronen *et al*., 2003).

In infected fish, large amounts of the virus are found in the kidneys and lesser amounts in the liver and brain as detected by immunofluorescence and by semi-quantitative PCR (Ronen *et al*., 2005). The same study reported that interstitial nephritis was detected as early as 2 days post-infection with increasing severity up to 10 days post-infection. In an earlier experimental study where real-time TaqMan PCR was used to quantify KHV DNA, the greatest amounts of viral DNA were found in the gill, kidney and spleen with virus genome equivalents of 10^8 to 10^9 per 10^6 host cells (Gilad *et al*., 2004). High levels of KHV DNA were also found in mucus, liver, gut and brain. Yuasa *et al*. (2005b) experimentally immersed common carp fingerlings in 10^3 $TCID_{50}$/ml at 23°C and subsequently detected the virus in several organs including the gills from 3-40 days post-infection by PCR. However, the virus was not detected in any organ 60 days post-infection. The amount of the virus was higher, and peaked at 7-9 days, in the gills, fin, scales, kidney and intestine than in other organs. The virus could be isolated, in CCB cell cultures, from the gills and kidney 6-17 days post-infection but not after 20 days. Interestingly, KHV was detected in fish brain as long as 145 days after infection but was infectious only for up to 28 days (Yuasa *et al*., 2007).

Severe gill disease was associated with inflammation and loss of villi (Pikarsky *et al*., 2004). Other studies reported that the gills of infected fish develop necrotising branchitis, lamellar epithelial degeneration, focal areas of necrosis and exfoliation. Histologically, infected fish shows hyperplasia and fusion of the secondary gill lamellae (Tu *et al*., 2004a; Cruz-Lacierda *et al*., 2005). Intranuclear inclusions in the branchial epithelium may be observed. In addition, Ilouse *et al*. (2006) reported that the kidneys of infected fish exhibited heavy, interstitial, inflammatory infiltrates 6 days post-infection. This condition was associated with large cells having a foamy, distended cytoplasm and a few intranuclear inclusion bodies. By day 8, severe infiltration developed and was accompanied by a feathery degeneration of the tubular epithelium in many nephrons. Mild, focal inflammation was also noted in the livers and brains.

Temperature is a critical factor in the pathogenesis of KHV infections. KHV induces infection/mortalities at temperatures of 18-25°C. Experimental infection showed that exposure of healthy fish to KHV at 22°C can cause up to 82% mortality within 15 days (Ronen *et al*., 2003). Furthermore, fish exposed to KHV at 20-24°C for 3 days then transferred to non-permissive temperatures survived the infection. However, fish held at 13°C for 30 days and shifted to 22-24°C developed disease and rapid onset of mortality. In another study, Yuasa *et al.* (2008) showed that common carp experimentally exposed to KHV at 16°C, 23°C and 28°C died from infection at 21-54, 5-20 and 7-14 days post-virus exposure (dpe), respectively. Moreover, using cohabitation with naïve koi carp, infected carp were shown to continuously shed the virus for 34 days (7-40 dpe) at 16°C, for 14 days (1-14 dpe) at 23°C, and for 12 days (3-14 dpe) at 28°C.

The virus has been isolated from KHV-infected common/koi carp in different carp cell lines: koi fin (KF-1), koi fin (KFC), common carp brain (CCB) and koi tail (KT-2) in USA, Israel, Germany, Indonesia, Japan, Singapore and Taiwan (Hedrick *et al*., 2000; Neukirch and Kunz 2001; Sano *et al*., 2004a, Lio-Po and Orozco 2005; Ilouse *et al*., 2006; NACA and FAO 2008). The KHV-infected cells cultured at 20-25°C demonstrate typical cellular vacuolation. However, the vacuolated cells reverted to normal morphology and plaques disappeared following shift to the non-permissive temperature, and reappeared after transfer back to permissive temperature (Dishon *et al*., 2007).

The virulence of the virus appears to be more potent in 14 g common carp (dying 6 to 9 days post exposure) than in 6 g fish (succumbed in 10 to 14 days) at 20-24°C (Lio-Po *et al*., 2006). Similarly, recent comparative infection studies showed that three-day old common carp fry (mean TL: 7.5 and 8.7 mm) exposed to KHV were not susceptible to disease while 69-100% mortalities were observed in common carp juveniles (mean TL: 13.8 and 29.2 mm) reared at 24°C (Ito *et al.,* 2007b).

Variation in susceptibility among different carp strains has been reported. In Europe, the ghost carp shows higher sensitivity to the virus than koi carp. In Japan, the indigenous strain of common carp shows higher sensitivity to the virus than the Eurasian strain of common carp or koi carp (Ito *et al*., 2007a).

The virus can remain latent in the host for long periods of time, becoming active only at permissive temperatures. Thus, the virus persists in "carriers" which demonstrate no clinical signs of the disease. Active KHV infection recurred in recovered fish once they were stressed. Thus, even after 7 months post-infection the virus can be reactivated. Moreover, the virus can persist in the brain of its fish host for at least 360 days (Yuasa *et al*., 2007). Latent KHV can be reactivated by increasing the water temperature from non-permissive to permissive temperatures. Such reactivation was confirmed by virus transmission and detection to naïve carp cohabited with infected carp at 150 days following the initial infection. Moreover, the virus was transmitted horizontally from KHV-infected fish to naïve koi or common carp via intraperitoneal injection or by bath exposure at permissive temperatures (18-25°C). Moreover, once infected, a pond will lose the majority of its fish within days, and no treatment is currently licensed.

In addition, non-specific secondary infections of bacterial, parasitic and fungal origin may be associated with KHV infections. For instance, secondary gill infections attributed to *Flavobacterium columnare* and *Aeromonas* spp. have often been associated with KHV infection (Sunarto *et al*., 2005b).

DIAGNOSIS

Several techniques have been used for detecting KHV in fish hosts. Among these are one-step and nested PCR, loop-mediated isothermal amplification (LAMP), *in situ* hybridization, cell culture, histopathology, transmission electron microscopy (TEM), enzyme-linked immunosorbent assay (ELISA), and bioassay in healthy, naïve, susceptible fish.

PCR is a more specific and sensitive method for KHV detection (Fig. 4) using the Sph and 9/5 primers (Gray *et al.,* 2002; Gilad *et al.,* 2002). More recently, Yuasa *et al.* (2005a) improved the PCR protocol using the *Sph* I-5 primer set that reduced non-specific reactions and reduced the reaction time by almost half. At about the same time, Bercovier *et al.* (2005) developed the TK PCR primer set to similarly improve the technique. In addition, a nested PCR has been developed with more sensitivity for KHV detection (Liu *et al.*, 2002; El-Matbouli *et al.*, 2007). Commercial PCR kits are already available in the market. A reflection of how widespread the use of PCR for KHV detection has become is illustrated by the broad participation in the international inter-laboratory proficiency test organised by Way *et al*. (2008) which involved 21 laboratories from 19 countries in 2006 and 32 laboratories from 27 countries in 2007. Results from these ring tests indicated that the Bercovier-TK (1) and modified Gray SpH (2) primer sets were the most robust for detection of KHV DNA. In addition, real-time PCR, useful for the quantitative estimation of the KHV virus, has been developed using the TaqMan PCR method (Gilad *et al.*, 2004).

More recently, the LAMP method was developed and reported to be as sensitive as PCR and to be more rapid than PCR for the detection of KHV. LAMP methods used to date include TK LAMP, 9/5 LAMP and Sph LAMP (Gunimaladevi *et al*., 2004; Soliman and

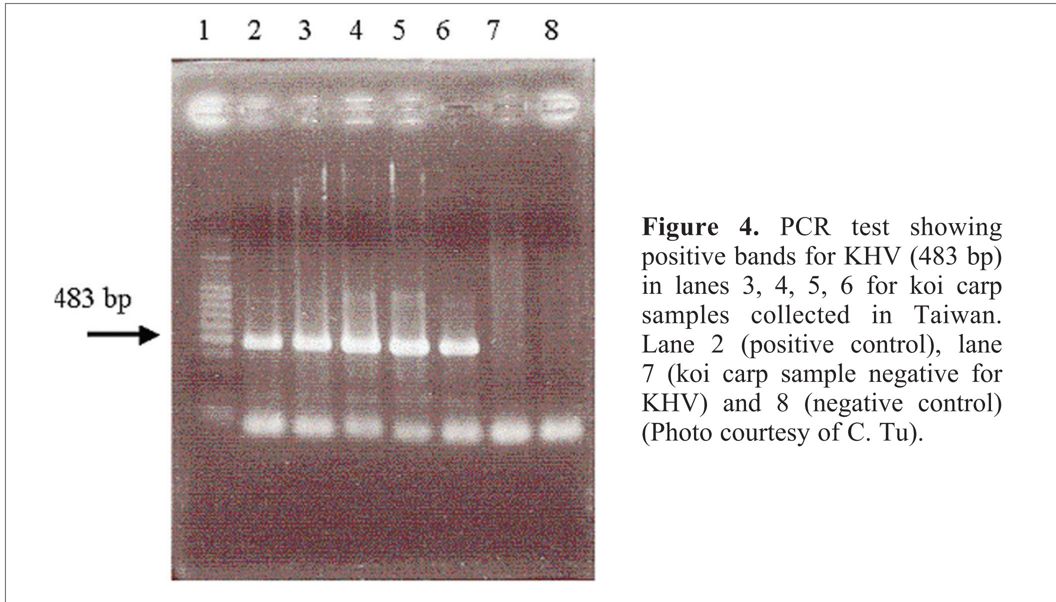

Figure 4. PCR test showing positive bands for KHV (483 bp) in lanes 3, 4, 5, 6 for koi carp samples collected in Taiwan. Lane 2 (positive control), lane 7 (koi carp sample negative for KHV) and 8 (negative control) (Photo courtesy of C. Tu).

El-Matbouli, 2005; Yoshino *et al.*, 2006). Other DNA based detection methods include *in situ* hybridization (Haenen *et al.*, 2004) and the RT-PCR targeting the mRNA terminase (Yuasa *et al.*, 2007).

The virus can be isolated in susceptible fish cell lines such as KF-1, KFC, KT-2 or CCB. Tissue filtrates are prepared from the gills, kidney, spleen or leukocytes by homogenization, centrifugation and filtration through 0.45 micron membrane filters. Inoculated cells are incubated at 20-25°C for 7-14 days. Infected cells develop cytopathic effect (CPE) consisting of typical cellular vacuolation (Fig. 5). The survival of KHV in infected CCB cells was maintained for 30 days at 30°C (Dishon *et al.*, 2007).

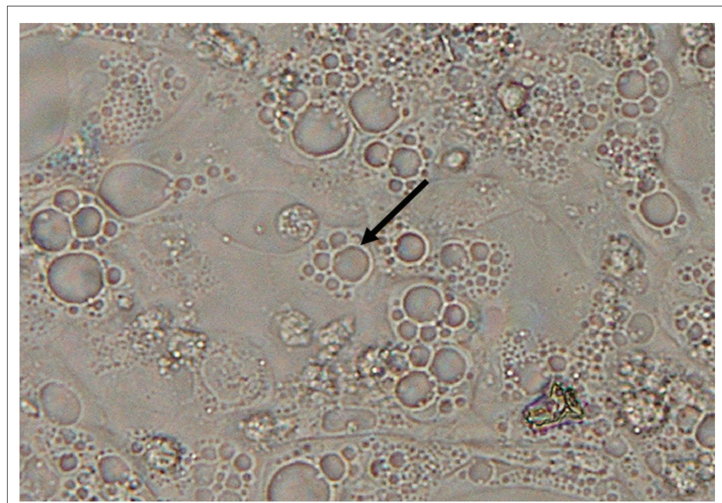

Figure 5. Cultured KF-1 cells inoculated with KHV showing typical vacuolations (arrow).

Histological examination of gills, liver, spleen and kidney of affected fish is also useful for diagnosis. The presence of severe gill hyperplasia with lamellar fusion and epithelial necrosis with prominent nuclear swelling and eosinophilic, intranuclear inclusions is diagnostic for KHV infection (Tu *et al*., 2004a; Cruz-Lacierda *et al*., 2005). Necrosis may also be observed in the liver, spleen and kidney parenchymal cells.

Electron microscopy used for visualizing KHV virions (Hedrick *et al.,* 2000; Miwa *et al*., 2007) is another useful direct diagnostic method. Indirect methods such as the enzyme-linked immunosorbent assay (ELISA) allows detection of antibodies to KHV in the serum of koi and common carp previously exposed to the virus (Adkison *et al*., 2005).

Where laboratory facilities for Level III Diagnosis are unavailable, the presence of KHV can also be confirmed by bioassay (Lio-Po *et al*., 2009). Tissue filtrates prepared from KHV-infected fish are injected intraperitoneally to healthy koi or common carp which are maintained at the permissive temperature (23-28°C) for at least 3 weeks. Development of typical clinical signs of KHV infection in the naïve fish is a presumptive indication of the presence of the virus. Confirmatory diagnosis is achieved by virus isolation in cell culture and identification by PCR. However, for expensive koi carp (where it is important to keep the fish alive), establishment of the KHV infection status can be determined by a modified "Sentinel" bioassay. This is a non-destructive technique wherein an expensive, koi carp, suspected of being infected with KHV, is cohabited with known KHV-free common carp at 23-28°C for 2 to 4 weeks. The development of KHV clinical signs in the naïve common carp indicates KHV infection in the suspect koi carp. Conversely, if the common carp remain negative for KHV, then the expensive koi carp with which it was cohabited is also considered KHV-free.

PREVENTION AND CONTROL

The impact of KHV is very significant in the koi-keeping/breeding community because of its high pathogenicity. Unfortunately, there is no effective drug for KHV disease. The aquarium fish trade, especially of koi carp, most likely played a significant role in the transboundary movement of the virus. The annual fish trade shows featuring live ornamental fish sourced from many countries have contributed to the transmission of fish pathogens (Lio-Po, 2007). For example, there is a documented case of illegally imported live koi carp that was intercepted by the fish quarantine staff at Ninoy Aquino International Airport in Manila, Philippines in September 2004. Five days after stocking the fish in a contained facility, the koi developed clinical signs and tested positive for KHV by PCR. All koi died after two weeks in the holding tank (Somga *et al*., 2010). Hence, health certification, quarantine and testing of imported koi or common carp for KHV should be instituted by the importing country before the imported fish gain entry into fish farms. In fish farms, biosecurity measures must be in place to mitigate against disease introduction, to ensure containment and to prevent dissemination of KHV among existing farm stocks.

In Israel, experimental cohabitation of naïve koi carp with KHV-infected fish induced the production of anti-KHV antibodies among exposed naïve carps (Ronen *et al.*, 2003). These findings led to studies on the development of a live, attenuated KHV vaccine that can induce high antibody titers in test fish after 7 dpi and peaks at 21 dpi (Ronen *et al.*, 2003 and 2005; Perelberg *et al.*, 2005; Ilouse *et al.*, 2006). Fish were immersed in 10 to 100 PFU/ml attenuated virus for 40 min followed by incubation at the permissive temperature for an additional 48 to 72 h (Perelberg *et al.*, 2005). Vaccinated fish survived subsequent challenge with the virus yielding a relative percent survival (RPS) of 80-95%. Field applications of the vaccine in carp in Israel from 2003 to 2007 resulted in increased carp production to 16,000 tonnes in 2007 compared to 8,000 tons in 2002 when the KHV vaccine was not in use. Moreover, protective immunity after vaccination extends for at least 8 months among vaccinated fish (Ilouse *et al.*, 2006). This vaccine is currently commercially available (M. Kotler, *pers. comm.*).

In Japan, Yasumoto *et al.* (2006) used a formalin-inactivated KHV vaccine entrapped within the liposomal membrane that was experimentally sprayed on dry pellets before feeding to common carp. The vaccinated fish yielded an RPS of 74.4% and 65% upon challenge with its homologous virus 22 days after vaccination. However, no further studies on field testing or commercialization of the vaccine were reported.

The viability of KHV under non-permissive conditions may also indicate a potential method to control KHV infections. For instance, cohabitation of fish with KHV infected fish for 3-5 days at permissive temperatures of 22-23°C and then transferred to ponds with water temperature of approximately 30°C for 30 days then re-challenged with the virus had significantly reduced mortalities of 39% compared to the control fish (not cohabited with sick fish) kept at 22°C and then challenged with the virus yielding 82% mortalities within 15 days (Ronen *et al*., 2003).

The infectious titre of KHV kept in environmental water was significantly reduced within 3 days (Shimizu *et al.*, 2006). Experiments on survivability of KHV indicated that the virus cannot tolerate temperatures of 35°C even for just 24 h (Lio-Po *et al*., 2006). Moreover, at 20 to 30°C, the virus remains viable up to 3 days while at -5 to 0, 4 and 15°C up to 5 days. However, KHV stored at -80°C has maintained its viability even one year after storage. Moreover, KHV was shown to be completely inactivated when exposed to UV irradiation (4.0 x 10^3 µWs/cm^2, 50°C for 1 min, 200 mg/L iodophor, 60 mg/L benzalkonium chloride or 30% ethyl alcohol for 20 min (Kasai *et al*., 2005).

Successive outbreaks caused by KHV among several different countries are indications of the transboundary movement of this emerging viral pathogen (Lio-Po 2007). Moreover, a further indication of the global significance of this disease is its recent (since 2007) addition to the list of notifiable diseases of the World Organization of Animal Health or the Office International des Epizooties (OIE).

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Random amplified polymorphic DNA (RAPD): a powerful method to differentiate *Streptococcus agalactiae* strains

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ABSTRACT

Streptococcus agalactiae from five different locations comprised of Pedu Lake (Kedah, Malaysia), Kenyir Lake (Terengganu, Malaysia), Pergau Lake (Kelantan, Malaysia), NTL Farm (Kelantan, Malaysia) and Wawasan Farm (Kelantan, Malaysia) were sampled and characterized by the API System. In order to achieve strain typing, all of the isolates were investigated by random amplified polymorphic DNA-PCR (RAPD-PCR). The RAPD-PCR technique was used to detect the genetic diversity of the five different sampling sites in Malaysia. Initially, 20 RAPD primers (Operon, USA) were used for screening before suitable primers that showed optimal level to be used later. Six 10-mer RAPD primers revealed significant bands ranging from 400-5000 bp. The primers were OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20. The genetic identity and genetic distance were calculated to reveal the diversities among the five different locations of sampling sites by using RAPD Distance software before a phylogenetic tree was constructed. The results indicated that the genetic variation from different locations is relatively high. Four out of five places exhibited a very close relationship with each other but a relatively distant relationship with Pergau Lake isolates.

Keywords: *Streptococcus agalactiae,* random amplified polymorphic DNA (RAPD), phylogenetic tree, tilapia.

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INTRODUCTION

Streptococcal disease in fish was first reported in 1957, in cultured rainbow trout in Japan, (Hoshina *et al*., 1958). Since then, numerous other species of fish have been found susceptible to the infection, including tilapia (Plumb, 1997). In a study conducted by Ferguson *et al*. (1994), *Streptococcus agalactiae* was shown to be more aggressive than any other environmental bacterium, where mortality may reach 100%. This indicates a need for studies on streptococcal infection in tilapia in Malaysia, particularly on the characterization of *S. agalactiae* isolated from diseased tilapia. This may lead to a better understanding of the epidemiology and assist in formulating better management of this pathogenic bacterium especially in cage-cultured tilapia.

A surveillance study by the National Fish Health and Research Centre (NaFisH) Penang, Malaysia, found that *S. agalactiae* was frequently isolated from cases of high mortality of cage-cultured tilapia in Kenyir and Pergau in 2002 to 2003. This incidence was found to be related to seasonal changes that affect the water quality parameters leading to unbalance physiological conditions of the fish. Alpha and beta hemolytic types of *Streptococcus* spp. were isolated.

Streptococcus agalactiae is a Group B *Streptococcus* (GBS), typically Gram-positive, cocci in chains and displays beta-hemolysis when cultured on a blood agar plate. The polysaccharide anti-phagocytic capsule in this bacterium is the main virulence factor that causing a disease of *Streptococcus* septicemia in tilapia. Therefore, the objective of this study is to determine the molecular epidemiological pattern of streptococcal infection of tilapia in Malaysia using molecular characterization of randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). The PCR was used to amplify DNA samples of *S. agalactiae* with short oligonucleotide primers, which annealing process take place randomly throughout the genome of the isolates (Snowdon and Langsdorf, 1998).

MATERIALS AND METHODS

Bacterial isolates and growth conditions
Isolates of *S. agalactiae* were obtained from NaFisH, Penang. The isolates were collected from Kenyir Lake, Pergau Lake, Pedu Lake, NTL Farm and Wawasan Farm in Malaysia between 2003 and 2005. All isolates were cultured onto blood agar plates within 18 to 24 hr and incubated at 30°C. The bacteria were sub-cultured into brain heart infusion broth (BHIB) (MERCK, Germany) and incubated at 30°C on a shaker for 18 hr. The bacteria were harvested by centrifugation at 3,500 *g*, 4°C for 10 min.

DNA extraction
DNA from the isolates was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

RAPD-PCR

RAPD-PCR was performed to detect the genetic diversity of five different sampling sites of *S. agalactiae* in Malaysia. Six 10-mer RAPD primers (Operon, USA) were screened out of 20 (OPA1 - OPA20). The primers are as in Table 1.

Table 1
Primers use in RAPD-PCR of *S. agalactiae*

No.	Primers	Primer Sequence
1.	OPA 3	5’- AGT CAG CCA C-3’
2.	OPA 4	5’- AAT CGG GCT G -3’
3.	OPA 5	5’- AGG GGT CTT G -3’
4.	OPA 9	5’- GGG TAA CGC C -3’
5.	OPA 11	5’- CAA TCG CCG T -3’
6.	OPA 20	5’- GTT GCG ATC C -3’

Polymerase chain reaction

All PCR’s were carried out in a thermal cycler (Eppendorf, Germany) in 25 µl reaction volumes. The 0.2 ml thin-walled tubes were used to amplify the DNA of each reaction mixtures. Each reaction mixture contained 1.0 µg genomic DNA, 0.5 µl of 10 mM dNTPs mix, 2.0 µl of 25 mM $MgCl_2$, 2.5 µl of 10× PCR buffer (100 mM Tris-HCl (pH 8.8 at 25°C); 500 mM KCl; 0.8% Nonidet P40), 0.5 µl of *Taq* DNA polymerase (MBI Fermentas, USA) and 25 mM of each primers (First Base Laboratory, Malaysia). Standard amplification conditions were 94°C for 4 min followed by 44 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 1 min and the reaction was completed by a final extension step of 72°C for 10 min.

Gel electrophoresis

A gel consisted of 1% agarose in 1× TBE running buffer was prepared. Following that, the PCR product solution was mixed with 3 µl of 6× loading dye solution (Fermentas, Lithuania) and electrophoresed at 100 V for 45 min in a MT-108 Wide Mini Horizontal Gel Electrophoresis System. The gels were stained with 1 µg/ml ethidium bromide (Sigma, USA) for 30 min and visualized with an ultraviolet transilluminator.

Data analysis

Target DNA amplicon were scored (1 for band visible, 0 for no band visible) for each suitable marker (bp). Faint bands were considered. Results were then entered in NTedit 1.2a software according to the markers selected. Construction of a phylogenetic tree was done using NTSYSpc 2.10j (Numerical Taxonomy and Multivariate Analysis System version 2.1j) software.

RESULTS

By using six significant primers (OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20), results revealed that the bands ranged from 4000 bp (OPA20) to ~ 300 bp (OPA5). OPA5 produced the most DNA fragments (7 fragments). Fig. 1 showed RAPD amplification of *S. agalactiae*

using OPA1 - OPA20 primers, which highlighted that different primers synthesize different molecular weight of bands. Primers that gave the most number of synthesized bands and the highest density of fragment bands (referring to the bright color on the agarose gels) were chose to use on the rest of the isolates. Figs. 2 - 7 showed RAPD amplification of *S. agalactiae* using OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20. As referred to Figs. 2 - 7, some isolates looks similar but the faint band is the key that differentiates each isolate tested.

RAPD analysis

The band migration distances for each lane were determined and the molecular size of each fragment was calculated from migration distances. By using NTedit 1.2a, all data were entered as shown in Tables 2 - 7. The phylogenetic tree for the 5 different sampling

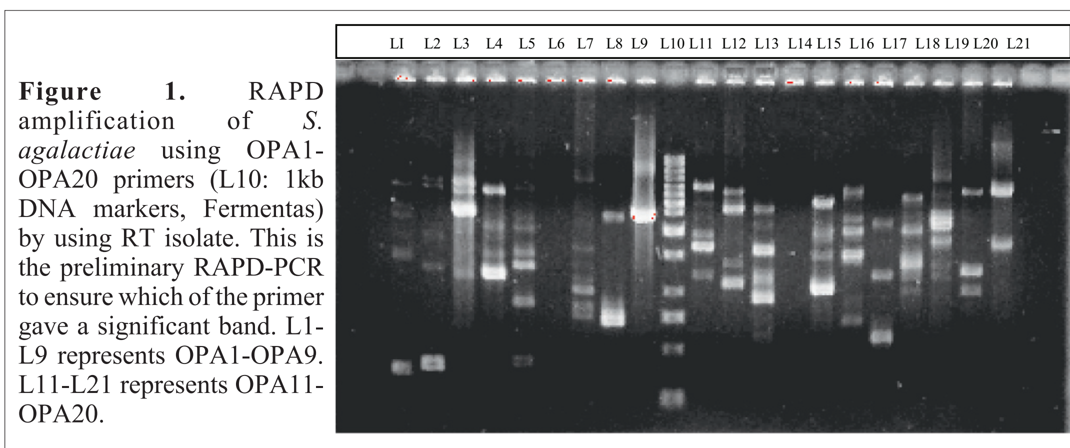

Figure 1. RAPD amplification of *S. agalactiae* using OPA1-OPA20 primers (L10: 1kb DNA markers, Fermentas) by using RT isolate. This is the preliminary RAPD-PCR to ensure which of the primer gave a significant band. L1-L9 represents OPA1-OPA9. L11-L21 represents OPA11-OPA20.

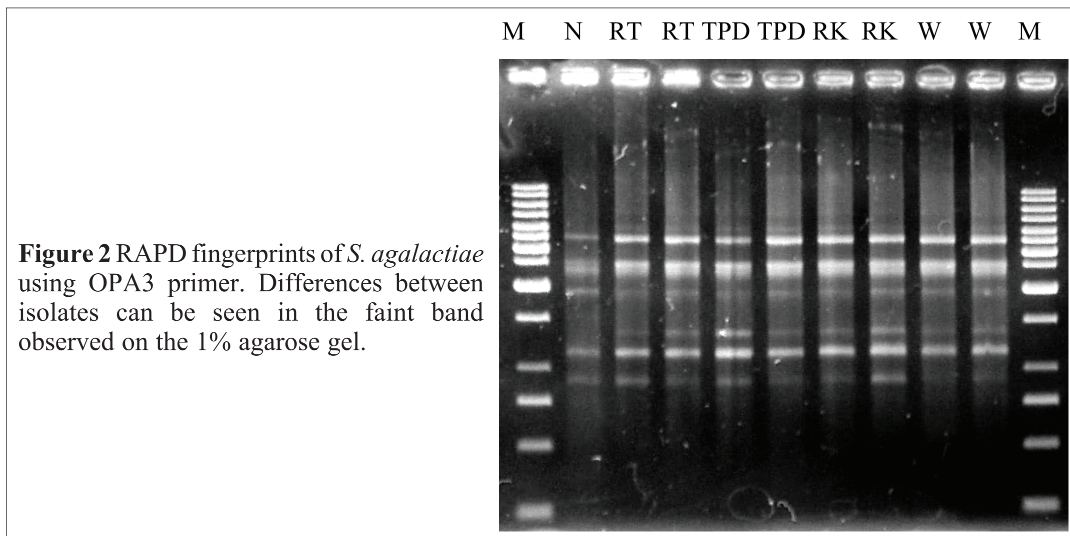

Figure 2 RAPD fingerprints of *S. agalactiae* using OPA3 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

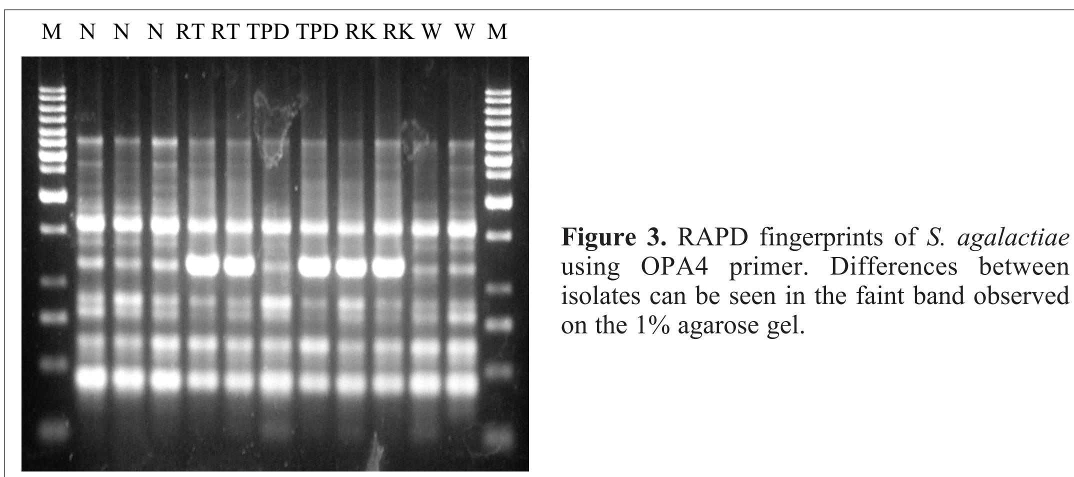

Figure 3. RAPD fingerprints of *S. agalactiae* using OPA4 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

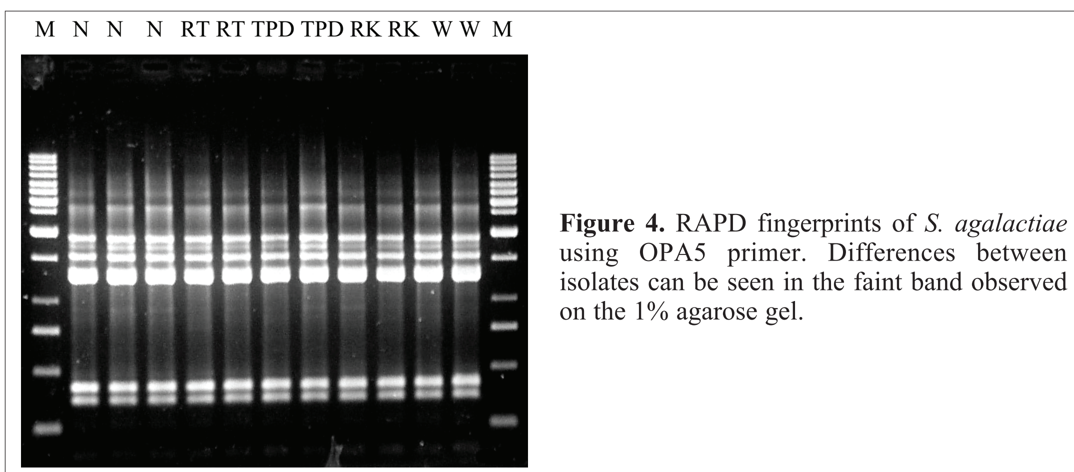

Figure 4. RAPD fingerprints of *S. agalactiae* using OPA5 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

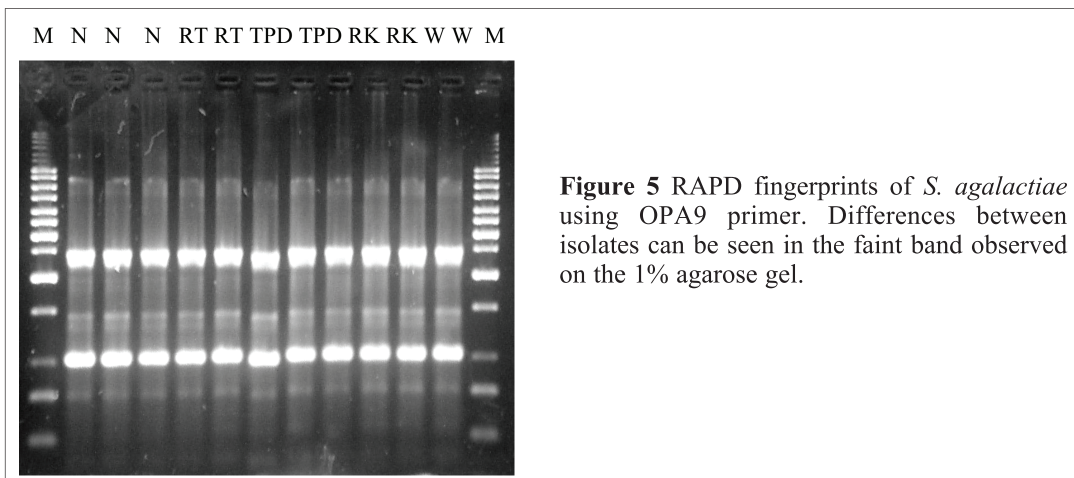

Figure 5 RAPD fingerprints of *S. agalactiae* using OPA9 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

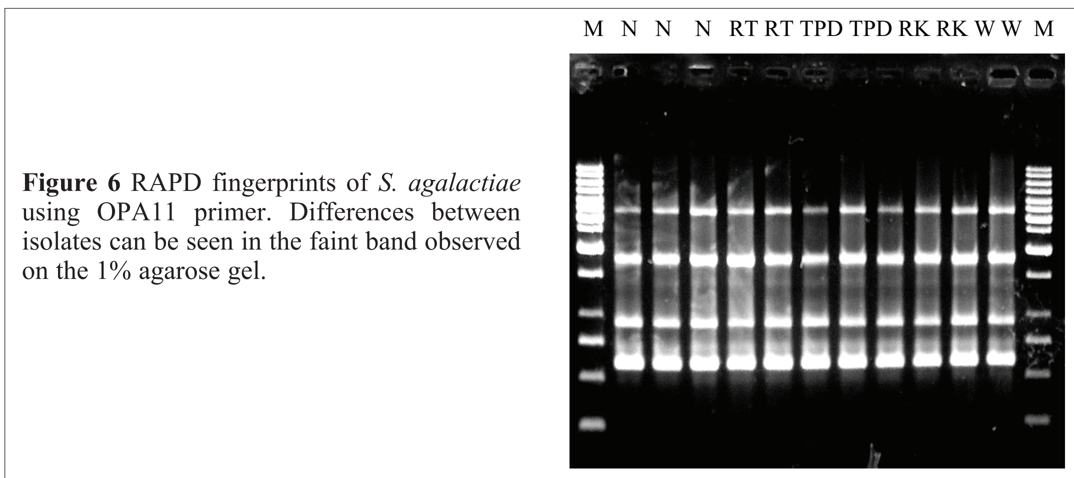

Figure 6 RAPD fingerprints of *S. agalactiae* using OPA11 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

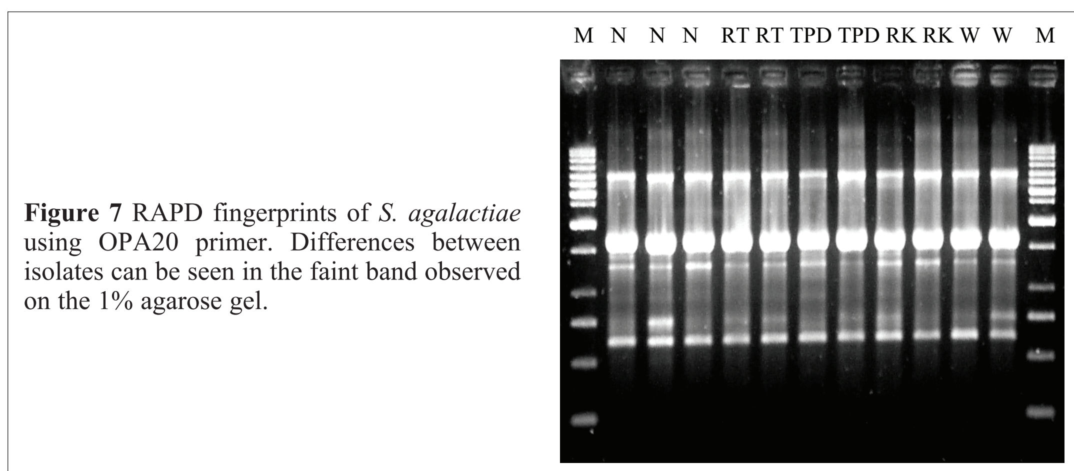

Figure 7 RAPD fingerprints of *S. agalactiae* using OPA20 primer. Differences between isolates can be seen in the faint band observed on the 1% agarose gel.

sites locations, Pedu Lake (TPD), Kenyir Lake (RT), Pergau Lake (RK), NTL Farm (N) and Wawasan Farm (W) was constructed. Cluster analysis generated two arbitrary groups. The phylogenetic tree (Fig. 8) revealed two major clusters. The first cluster showed two subclusters, which contained *S. agalactiae* isolated from four different sampling sites, while the second cluster showed that RK was totally different compared to the other *S. agalactiae* from the other sampling sites.

DISCUSSION

As shown in Fig. 8, the phylogenetic tree demonstrated that *S. agalactiae* isolated from RK are genetically different compared to the other *S. agalactiae* isolates. *S. agalactiae* isolated from RT, TPD, W and N were genetically similar as they are in the same cluster. However, *S. agalactiae* isolated from N were slightly different from the other even though they were in the same cluster. DNA sequencing is required to detect any slight mutation that may be present.

Table 2
Band Scoring Table for isolates using OPA3 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	1	1	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	1
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	1	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 3
Band Scoring Table for isolates using OPA4 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	1	1	1	1	1
0.60	1	1	1	1	1
0.40	1	1	1	1	1
0.30	0	0	0	0	0

Table 4
Band Scoring Table for isolates using OPA5 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	0	0	0	0	0
2.50	1	0	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	1	1	1	1	1
1.70	1	1	1	1	1
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	1	1	1	1	1
0.30	1	1	1	1	1

Table 5
Band Scoring Table for isolates using OPA9 primer

Band (kb)	**RT**	**RK**	**TPD**	**W**	**N**
4.00	0	0	0	0	0
3.50	0	0	0	0	0
2.50	1	1	1	1	1
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	1	1	1	1	1
1.00	1	1	1	1	1
0.80	0	0	0	0	0
0.75	0	0	0	0	0
0.60	0	0	0	0	0
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 6
Band Scoring Table for isolates using OPA11 primer

Band (kb)	RT	RK	TPD	W	N
4.00	0	0	0	0	0
3.50	1	1	1	1	1
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	1	1	1	1	1
1.40	0	0	0	0	0
1.50	0	0	0	0	0
1.25	0	0	0	0	0
1.00	0	0	0	0	0
0.80	1	1	1	1	1
0.75	0	0	0	0	0
0.60	1	1	1	1	1
0.40	0	0	0	0	0
0.30	0	0	0	0	0

Table 7
Band Scoring Table for isolates using OPA20 primer

Band (kb)	RT	RK	TPD	W	N
4.00	1	1	1	1	1
3.50	0	0	0	0	0
2.50	0	0	0	0	0
2.25	0	0	0	0	0
2.00	0	0	0	0	0
1.80	0	0	0	0	0
1.70	0	0	0	0	0
1.40	0	0	0	0	0
1.50	1	1	1	1	1
1.25	1	1	1	1	1
1.00	0	0	0	0	0
0.80	0	0	0	0	0
0.75	0	1	0	0	1
0.60	1	1	1	1	1
0.40	0	0	0	0	0
0.30	0	0	0	0	0

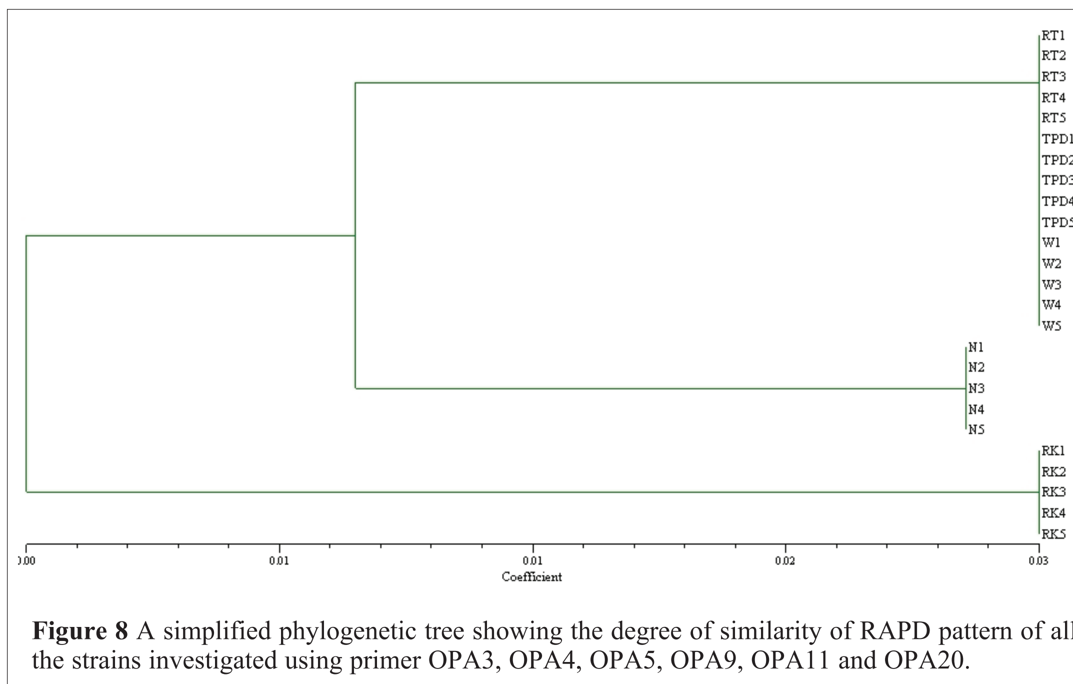

Figure 8 A simplified phylogenetic tree showing the degree of similarity of RAPD pattern of all the strains investigated using primer OPA3, OPA4, OPA5, OPA9, OPA11 and OPA20.

From the phylogenetic tree, geographical relations were not shown to be a factor because RT, RK, W and N are located at the west of Malaysia, while TPD is located at the east of Malaysia. So, the main factor may be the source of the fish obtained or perhaps it was the results of transposable elements, insertions or deletions in the DNA that need further sequencing for confirmation.

RAPD is one of the most powerful methods to differentiate *S. agalactiae* strains. Six of the twenty primers tested showed significant bands, which enabled construction of a phylogenetic tree. Interestingly, RAPD can also differentiate each *S. agalactiae* isolates within the species due to polymorphisms. Generally, if more isolates of *S. agalactiae* from various part of Malaysia were studied, the phylogenetic tree constructed will represent more of *S. agalactiae* strains in Malaysia.

In conclusion, RAPD is a useful method in analyzing complexes of species (Moschetti *et al.,* 1997; Martinez *et al.,* 1999). It has several advantages such as requiring very little genomic DNA, simple methodology, it is useful when there is no prior knowledge of sequencing data and it does not require the use of hazardous materials. In a lactobacillus study, the RAPD techniques have been extensively used in the strain and intraspecies typing of lactic acid bacteria (Sylviya *et al.,* 2008). On the other hand, Pulsed Field Gel Electrophoresis (PFGE) and Amplified Fragment Length Polymorphism (AFLP) may be used in order to discriminate better between the isolates.

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Digenean trematodes of cultured grouper (*Epinephelus coioides* and *E. bleekeri*) in Khanh Hoa Province, Vietnam

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ABSTRACT

Groupers are among the most important fish cultured in Vietnam and, recently, diseases due to parasites have led to massive losses for the aquaculture industry. To date, little research has been conducted on parasitic diseases of marine fish in Vietnam. To address this, 156 specimens, collected from January to December 2006, of pond- and cage-cultured *Epinephelus coioides* and *E. bleekeri,* with size ranging from 85 – 500 mm (mean = 286.8 ± 96.4 mm) were examined. Results show that cultured grouper in Khanh Hoa Province, Vietnam were infected with adults of at least 5 digenean species. Three intestinal digeneans (*Prosorhynchus epinepheli, P. pacificus,* and *Helicometra fasciata*), one from the stomach (*Erilepturus hamati*), and one from the skin (*Transversotrema patialense*) were identified. Unidentified dydimozoids and metacercaria were found in the musculature. *Prosorhynhcus pacificus* had the highest prevalence of infection. There was a statistically significant difference between the prevalence of infection in *P. pacificus* in pond- and cage-cultured *E. coioides* (61% (20/33) prevalence in cage-cultured fish; 27% (9/33) in pond-cultured fish). *Prosorhynhcus epinepheli* is a first record for *E. bleekeri; P. pacificus* is a first record for *E. coioides* in Vietnam; *E. hamati* and *H. fasciata* are first records for *E. coioides and E. bleekeri; T. patialense* is a first record for Vietnam and for *E. bleekeri.* This is the first time that the Family Didymozoidae has been recorded in grouper in Vietnam, and a first record in *E. bleekeri*. The difference in intensities of infection of

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P. epinepheli and metacercariae in cage- and pond-cultured *E. bleekeri,* of *P. pacificus* and metacercaria in cage- and pond-cultured *E. coioides* were not statistically significant. Data on intensities of infection of the other digeneans were insufficient to be tested for statistical significance.

Key words: grouper, Vietnam, digenetic trematodes, parasites

INTRODUCTION

Groupers are widely cultured in South Europe and Asia, including Vietnam. In Vietnam, they have been used to replace black-tiger shrimp (*Penaeus monodon* Fabricius) and lobster (*Panulirus orgnatus* Fabricius, 1789) farming where disease outbreaks have made the culture of species economically unprofitable (Vo *et al*., 2008a). Grouper culture provides a good source of income to the poor communities in some provinces in northern and central Vietnam (Vo *et al*., 2008a). However, groupers are also sensitive to various diseases, and loss of grouper due to diseases has been estimated to cost many millions of dollars (Bondad-Reantaso *et al*., 2005).

In Vietnam, groupers have been found infected with numerous parasites (Vo *et al*., 2005, 2007a, 2007b, 2008a, 2008b, 2008c) including digenetic trematodes. Such parasites of marine and brackish water fish have been intensively studied in many countries. Previous to the above studies, the only English publication mentioning digeneans of Vietnamese grouper is the checklist of Arthur and Bui (2006).

While the impact of digeneans on their first intermediate hosts such as snails is clear (Muira and Chiba, 2007; Davies and Knowles, 2001), with specific exception of the blood dwelling sanguinicolids and some flesh dwelling didymozoids, their impact on fish has not often been observed, although some studies reported the impact of larval digeneans on fish (Olson and Pierce, 1997; Lauckner, 1984; Bristow, 1990). In order to evaluate the impact of digeneans to fish, it is first essential to know the composition, prevalence and intensity of infection of the digeneans in their hosts. The first aim of this study was to study these attributes in different fish hosts and of the same fish hosts collected from different culture systems. The second aim of this study was to assess if different culture systems cause differential effects to the parasitism of digeneans by comparing the prevalence and intensities of infection of the parasites.

MATERIALS AND METHODS

Collection sites

Epinephelus coioides Hamilton, 1822*,* and *E. bleekeri* Vaillant, 1878, were collected live in ponds from Cua Be Estuary and Cam Ranh Bay, and from cages in Cua Be Estuary, Nha Trang and Van Phong Bays, Khanh Hoa Province, Vietnam, between January and December, 2006. The ponds had a surface area of 1,500-3,000 m^2, and were 1-1.5 m in depth. All the ponds were located in tidal zones. The ponds in Cua Be Estuary were fed by

brackish water (with salinity ranging between 10 and 27 ‰), while the ponds in Cam Ranh Bay were fed by marine water (with salinity ranging between 31 and 33 ‰). The fish density in the ponds was about 3-5 fish/m^2 of the pond bottom areas. The cages used for rearing fish were floating net cages. Each cage had a surface area of approximately 9 m^2 and was 3-4 m deep and they were often linked together in groups. The cages in Cua Be Estuary were in brackish water (with salinity ranging between 10 and 27 ‰), while the cages in Nha Trang and Van Phong bays were in marine water (with salinity fluctuating between 31 and 33 ‰). Fish density in the cages was from 5-100 fish/m^2 of the cage bottom area. Fish in the ponds and cages were fed daily with trash fish, snails, bivalves, and crustaceans.

Fish were transported in styrofoam boxes with water from the original environment with aeration, from collection sites to the Research Institute for Aquaculture No.3 in Nha Trang. They were then kept in cement tanks with marine water (salinity between 30-32 ‰) continual flow through before being examined for parasites.

Each fish specimen was identified using Heemstra and Randall (1993) before parasitic examination. The number of each fish species collected from each sampling site, the length, weight (mean +/- standard deviation) are given in Table 1.

Examination for parasites

Fork length and body weight of each fish specimen were measured. Fins were cut and examined under a stereomicroscope for metacercariae. Skin was scraped and placed on glass slide for digeneans. Each fish was then dissected and its internal organs were placed in separate Petri dishes containing saline solution. Each organ was examined using stereomicroscope for parasites. The gut and stomach were dissected and examined by eye and then under a stereomicroscope. The flesh was pressed using glass pieces and observed under a stereomicroscope.

Identification and enumeration of parasites

Fresh specimens of digeneans collected were photographed, fixed, drawn, and measured for identification.

Table 1

Length and weight of cage and pond cultured *E. coioides* and *E. bleeker.* The minimum and maximum values are given in parenthesis.

	Cage cultured fish		**Pond cultured fish**	
	E. coioides (n=33)	*E. bleekeri* (n=62)	*E. coioides* (n=33)	*E. bleekeri* (n=28)
Mean length (mm)	351.6±41.0 (265.0-400.0)	256.5±86.7 (115.0-380.0)	327.8±110.1 (85.0-500.0)	227.9±76.3 (110.0-335.0)
Mean weight (g)	644.8±253.7 (187.0-1055.0)	309.5±246.4 (20.0-760.0)	709.2±448.5 (6.0-2000.0)	216.8±208.4 (13.6-650.0)

Data analysis

Standard parasitological parameters followed the recommendations in Bush *et al.* (1997). The prevalence of infection of each trematode species collected from cage- and pond-cultured host species was compared using a Chi-square test with Yates correction being employed if 5 or more fish were infected. When less than 5 fish were infected, the Fisher-exact test was used. The intensities of infection of the parasites between cage- and pond-cultured fish of the same species were compared using the Krusskal Wallis test, using a significance level of $\alpha = 0.05$.

RESULTS

The composition and the prevalence of infection of the parasites are given in Table 2. Five digeneans were identified to the species level. Adult trematodes include three species found in the intestines (*Prosorhynhcus epinepheli* Yamaguti, 1939; *P. pacificus* Manter, 1940; and *Helicometra fasciata* Rudolphi, 1819), one species found in the stomach (*Erilepturus hamati* (Yamaguti, 1934) Manter, 1947), and one species found in the skin (*Transversotrema patialense* Soparkar, 1924). An unidentified didymozoid in the flesh and unidentified metacercaria types in the fins were also found.

Table 2

Prevalences (P) and intensities (I) of infection of trematodes of cage- and pond-cultured *E. coioides* and *E. bleekeri.* The minimum and maximum values are given in parenthesis; *only 1 fish infected.

Parasites	Cage cultured fish				Pond cultured fish			
	E. coioides		*E. bleekeri*		*E. coioides*		*E. bleekeri*	
	P (%)	I	P (%)	I	P (%)	I	P (%)	I
Prosorhynchus epinepheli	0.0		6.4	30.8 ±44.1 (1; 95)	0.0		21.4	111.8 ±175.7 (1; 463)
Prosorhynchus pacificus	60.6	9.8 ±11.3 (1;39)	0.0		27.3	4.8 ±3.2 (2; 11)	0.0	
Erilepturus hamati	0.0		3.2	1.0 ±0.0 (1; 1)	3.0	1*	0.0	
Helicometra fasciata	3.0	2*	1.6	1*	0.0		7.1	1.5 ±0.7 (1; 2)
Transversotrema patialense	0.0		3.2	1.0 ±0.0 (1; 1)	0.0		0.0	
Didymozoidae	3.0	2*	1.6	1*	3.0	5*	0.0	
Metacercariae (in the fins)	6.1	1.3 ±0.6 (1; 2)	2.4	5.0 ±6.4 (1; 20)	9.1	3.7 ±3.8 (1; 8)	25.0	22.3 ±35.8 (1; 101)

A brief description of the parasites collected are shown below:

1. *Prosorhynhcus epinepheli*: body (1,150 – 1,750) x (350 – 750) µm; rhynchus (270 – 350) x (260 – 330) µm; gonads near midbody; testis slightly diagonal; vitellerium with 20-23 follicles, forming an arch between the rhynchus and stomach. This species was found in both cage- and pond-cultured *E. bleekeri*.
2. *Prosorhynchus pacificus*: body (1,100 – 2,850) x (360 – 1250) µm; rhynchus (220 – 400) x (200 – 410) µm; gonads near midbody; testis tandem; vitellerium with 18 – 24 follicles, forming 2 lateral lines from front testis up to near the rhynchus. This species was found in both cage- and pond-cultured *E. coioides*.
3. *Erilepturus hamati*: body (1,100 – 2,525) x (340 – 1,000) µm; ecsoma present. This ecsoma can protrude or withdrawn into the body; ventral sucker much larger than oral sucker; ovary posterior to testis; vitellarium ribbon-shaped. This species was found in cage-cultured *E. bleekeri* and pond-cultured *E. coioides*.
4. *Helicometra fasciata*: body 2.605 x 1.000 µm; ventral sucker much larger than oral sucker; egg with long tail; ovary lobes anterior to testis; two testes tandem. This species was found in cage-cultured *E. coioides* and in pond-cultured *E. bleekeri*.
5. *Transversotrema patialense*: body flattened dorsal-ventrally (480 – 1,000) x (310,0 – 692,0) µm; no oral sucker, ventral sucker small, rounded; testis big with 6-8 lobes; one ovary; eye-spots present; found under the scales. This species was found in cage-cultured *E. bleekeri*.
6. Unidentified didymozoid parasite: encapsulated; the capsulate is yellowish found in the flesh and operculum; body slender and whitish; ventral sucker large, in anterior half body. This parasite was found in cage-cultured *E. coioides* and *E. bleekeri* and pond-cultured *E. coioides*.
7. Unidentified metacercariae: the cysts were round or oval; some visible to naked eye; some are dark. Cysts were found in the fins of both *E. coioides* and *E. bleekeri* from both the cage- and pond-culture systems.

The highest (60.6%) and second highest prevalence of infection (27.3%) occurred among *P. pacificus* in cage- and in pond-cultured *E. coioides*, respectively. The prevalence of infection of other digeneans ranged between 1.6% (unidentified didymozoid in cage-cultured *E. bleekeri*) and 25.0% (unidentified metacercariae in pond-cultured *E. bleekeri*).

In each culture system, where both *E. coioides* and *E. bleekeri* were infected with the same digenean, *E. coioides* usually had the higher prevalence of infection. An exception was the prevalence of infection of the metacercaria in pond-cultured *E. coioides* and *E. bleekeri* where numbers were higher in the latter species.

The results of the comparison of the prevalence of each digenean in each fish species from cage- and pond-cultured system are given in Table 3. Results showed that only the prevalence of infection of cage- and pond-cultured *E. coioides* infected with *P. pacificus* were statistically significantly different (P = 0.01). In the other cases, the infection levels were either not sufficient to be compared or not statistically different.

Table 3
Comparison of the prevalence of trematode infected fish of the same species from cage and pond system.

Parasites	*E. coioides*			*E.bleekeri*		
	Cage cultured fish	Pond cultured fish	P value	Cage cultured fish	Pond cultured fish	P value
Prosorhynchus epinepheli	0/33	0/33	-	4/62	6/28	0.06
Prosorhynchus pacificus	20/33	9/33	0.01	0/62	0/28	-
Erilepturus hamati	0/33	1/33	1.00	2/62	0/28	1.00
Helicometra fasciata	1/33	0/33	1.00	1/62	2/28	0.22
Transversotrema patialense	0/33	0/33	-	2/62	0/28	1.00
Dydimozoidae	1/33	1/33	1.00	1/62	0/28	1.0
Metacercaria (in the fins)	2/33	3/33	1.00	15/62	7/28	0.85

The results of comparison of the intensities of infection of the digeneans in fish of the same species collected from cages and ponds are given in Table 4. This analysis showed that intensities of infection of the digeneans were not significantly different between cage- and pond-cultured hosts.

Table 4
Comparison of the intensities of digeneans in each fish species cultured in cage and pond systems (in the brackets are min and max of the respective values).

Parasites	**E. coioides**			**E.bleekeri**		
	Cage cultured fish	**Pond cultured fish**	**P value**	**Cage cultured fish**	**Pond cultured fish**	**P value**
Prosorhynchus epinepheli				30.8 ±44.1 (1; 95)	111.8 ±175.7 (1; 463)	0.67
Prosorhynchus pacificus	9.8±11.3 (1; 39)	4.8±3.2 (2; 11)	0.53			
Metacercaria (in the fins)	1.3±0.6 (1; 2)	3.7±3.8 (1; 8)	0.57	5.0±6.4 (1; 20)	22.3±35.8 (1; 101)	0.14

DISCUSSION

Digenean trematodes parasitise a wide-range of fish hosts, and are found in fresh, marine and brackish water fish and many species are found in numerous fish host species. They have been reported in 62 out of 159 species of groupers belonging to the sub-family Epinephelinea. Ninety percent of these host-parasite combinations have been reported only once or twice, and, no reports have suggested that groupers are free from trematode infections (Cribb *et al.*, 2002). This suggests that digenean trematodes in groupers have not been studied sufficiently.

In Vietnam, Arthur and Bui (2006) firstly gathered reports of digeneans in Vietnamese grouper. These authors mentioned digeneans parasitizing *Epinephelus moara* Bloch, 1793, *E. merra* Bloch, 1793, *E. sexfasciatus* Valenciennes, 1828 and *E. tauvina* Forsskåll, 1775. The cases reported by Arthur and Bui (2006) all occurred in the northern Vietnam, where *E. coioides* and *E. bleekeri* have not been reported as fish of natural distribution or in culture. Therefore, this study is the first to report digenean trematodes in *E. coioides* and *E. bleekeri* in Vietnam.

Groupers belonging to Family Serranidae have the fifth largest record of parasitic trematodes. The epinephelines ranks as the 10th largest number of host records for trematodes, harboring at least 147 trematode species (Cribb *et al*., 2002). This ranking may be higher if trematodes in grouper had received more study. Sixteen species of trematodes are restricted to, and shared among, epinephelines (Cribb *et al*., 2002). Leong and Wong (1990) found 3, 3, and 2 species of digeneans in cage-cultured *E. malabaricus* Bloch and Schneider, 1801 in Malaysia, Thailand and the Philippines, respectively. This study found 5 species, 1 family and an unidentified metacercaria in cultured *E. coioides* and *E. bleekeri*. Only *P. pacificus* was found by Leong and Wong (1990) and in the present study. This may be due to different fish host species examined.

The individual parasites identified in this study are discussed below:

1. ***Prosorhynhcus epinepheli***: This is a first record for this parasite in *E. bleekeri*, and it was not found in *E. coioides*. Arthur and Bui (2006) reported it from *E. tauvina, E. bruneus* Bloch, 1793, *E. sexfasciatus, E. merra* in Vietnam. While Cribb *et al.* (2002) listed 7 different hosts for this parasite, including *E. akaara* Temminck and Schlegel, 1842, *E. chlorostigma* Valenciennes, 1828, *E. cyanopodus* Richardson, 1846, *E. diacanthus* Valenciennes, 1828, *E. merra, E. quernus* Seale, 1901, *E. undulosus* Quoy and Gaimard, 1824. In addition, it was also found from *E. areolatus* Forsskåll, 1775 (Nahhas, Sey and Nakahara, 2006).
2. ***Prosorhynhcus pacificus***: This is the first record for Vietnam and for *E. coioides*. This species had the highest prevalence of infection among the digeneans in this study, infesting up to 60.6% and 27.3% of cage and pond cultured *E. coioides*, respectively. This is still lower than the prevalence of infection of 97.2% found by Leong and Wong (1988) in cage cultured *E. malabaricus* in Malaysia. The mean intensities of infection found in this study were also much lower, being 9.8% and 4.8% in cage cultured and pond cultured *E. coioides*, while Leong and Wong (1988) found 81.2% in cage cultured *E. malabaricus*. Chinabut *et al.* (1996) reported it from cultured *E. coioides* in Thailand. Cribb *et al.* (2002) present records for *E. malabaricus* and *E. tauvina*. Both *E. malabaricus* and *E. tauvina* are often misidentified with *E. coioides* (Heemstra and Randall, 1993).
3. ***Erilepturus hamati***: This species has been found in different fish species from various localities. It also has many synonyms; Bray *et al.* (1993) reported 25 synonyms of this species. It was reported in *E. malabaricus* in the Philippines (Arthur and Lumanlan-Mayo, 1997). Velasquez (1962) found this species in *Lates calcarifer* and described it as a new species, *Lecithochirium neopacificum*. Leong and Wong (1988) reported a

prevalence of infection of 15.7% of *E. malabaricus* and Chinabut *et al.* (1996) found this species in *E. coioides* in Thailand. This is the first record for this species in Vietnam, the first record for this parasite on *E. bleekeri*, and the first record on *E. coioides*.

4. ***Helicometra fasciata***: This is the first record for *E. coioides* and *E. bleekeri*. Arthur and Bui (2006) recorded it from *E. sexfasciatus* in Vietnam. Cribb *et al.* (2002) reported it from *Epinephelus fasciatus* Forsskåll, 1775*, E. merra*, and *E. quoyanus* Valenciennes, 1830.
5. ***Transversotrema patialense***: This is the first record for Vietnam and for *E. bleekeri*. Cribb, Bray and Barker (1992) reviewed the family Transversotrematidae and formally synonymised *T. koliensis, T. laruei, T. chackai, T. soparkari* with *T. patialense. Transversotrema koliensis* was originally described as *Cercaria koliensis* (Olivier, 1947). *Transversotrema laruei* was described by Velasquez (1958) from *Lates calcarifer* Bloch, 1790 in the Philippines, the fish being collected in brackish water. *Transversotrema chackai* was described by Mohandas (1973) from fresh water fish collected in the Chackai Canal in India. *Transversotrema soparkari* was described by Pandey (1971) from fish collected from India. *Transversotrema patialense* was described by Soparkar (1924) from India. Thus, *T. patialense* is found on fish either from fresh or/and brackish waters. In this study, this species was found in two cage cultured *E. bleekeri* from Cua Be Estuary, which is a brackish water area.
6. **Didymozoidae**: This is the first record from grouper in Vietnam, and the first record for *E. bleekeri*. In Vietnam, 5 species including *Didymozoon polymorphis* Oshmarin and Mamaev, 1963, *Monilicaecum ventricosum* Yamaguti, 1942, *Multtubovarium amphibolum* Mamaev, 1970, *Neomatanematobothrioides rachycentri* (Parukhin, 1969) Yamaguti, 1971 and *Torticaecum fenestratum* (Linton, 1907) Yamaguti, 1942 including some unidentified species belonging to this family have been reported from marine fish collected from Tonkin Gulf (Arthur and Bui, 2006). Globally, 7 genera of this family have been reported from epinepheline hosts, of which *Allonematobothrium* is most restricted to epinephelines with 5 out of 6 species being found only in this fish group (Cribb *et al*., 2002). Cruz-Lacierda *et al.* (2001) reported *Gonapodasmius epinepheli* in *E. coioides*.
7. **Metacercaria**: Larval trematodes have been studied intensively in many countries. Metacercaria have been reported from brackish and marine waters in some countries in Asia, particularly from South Korea (Joo, 1984; Seo *et al*., 1984; Chai and Lee, 2002). In Vietnam, Arthur and Bui (2006) reported records of digenean metacercariae found in some marine fish species such as *Drepane punctata*, *Ephippus orbis, Gerres philamentosus.* Vo *et al.* (2008b) reported a new record of the metacercaria of *Pygidiopsis suma*. In that paper, the authors also presented new records for *Heterophyopsis continua* and *Procerovum varium* metacercaria, while adult *H. continua* were reported by Nguyen (2000) from the sea gull (*Larus genei*).

Host-specificity has been studied for different parasites from different hosts and some studies have been conducted on the host-specificity of digenean trematode larvae; their hosts are usually mollusks (Möller and Anders, 1986). Generally, there are few references concerning fish host specificity of metacercaria or adult digeneans. Most of parasites

found in these studies seem to be able to parasitize different fish species. *Prosorhynhcus epinepheli* is found on many fish species (Cribb *et al.,* 2002; Arthur and Bui, 2006; Nahhas, Sey and Nakahara, 2006). *Erilepturus hamati* is found on different fish genera (Velasquez, 1962; Arthur and Lumanlan-Mayo, 1997; Bray *et al*., 1993). In the present study, it was found in both fish hosts. *Helicometra fasciata* is found in numerous species of *Epinephelus* (Arthur and Bui, 2006; Cribb *et al*. 2002). It was found in both hosts in this study with similar prevalence of infection. *Transversotrema patialense* is found in different fish species in both fresh and brackish waters (Soparkar, 1924; Olivier, 1947; Velasquez, 1958; Pandey, 1971; Mohandas, 1973). *Epinephelus bleekeri* is a new host record for this digenean. Didymozoidae and metacercariae were not identified to species level. Many species of different genera belonging to didymozoidae have been reported as parasites of different fish species, thus, making it difficult to discuss if these parasites are specific to any host.

In this study, the only evidence of host specificity is with *P. pacificus*. However, this species has also been reported from *E. malabaricus*, *E. tauvina*, and *E. coioides* by different authors and from *E. coioides* in this study. As referred to earlier, there has often been confusion in identifying these host species. In this study, due to the limited number of specimens used, it is not possible to confirm host specificity of this species and further investigation is needed to clarify this.

Muira and Chiba (2007) and Davies and Knowles (2001) reviewed the impacts of digenean trematode larvae on snails and bivalves. The most serious impact is the reduction of reproduction capability, including castration (Lauckner, 1984; Crew and Esch, 1986). They may also affect the burrowing ability of bivalves (Lauckner, 1984). Serious impact of digeneans on human and other animals has been confirmed by many studies (Hong *et al*., 1996; Chai, Murrell and Lymbery, 2005; Sohn and Chai, 2005). It has also been confirmed that the two fish hosts in this study carry metacercaria of zoonotic trematodes (Vo *et al*., 2008b). While adult digeneans found in the intestines of fish do not normally cause problems in fish (Schäperclaus, 1986), Lauckner (1984) indicated that a single metacercaria of digenean trematode is sufficient to kill a fish larva. Ogawa, Nakatsugawa and Yasuzaki (2004) reported a case of gross pathology caused by infection with digenean trematode metacercaria in cyprinid fish (*Zacco platypus* Temminck & Schlegel, 1846 and *Squalidus chankaensis chankaensis* Dybowski, 1872) in Japan; bucephalids were believed to be the disease causing agents. Olson and Pierce (1997) reported cartilage proliferation phenomenon in steelhead trout (*Oncorhynchus mykiss* Walbaum, 1792) collected from Willamette River, United States of America; members of the families Heterophyidae and Cryptogonimidae were suggested to be the agents. Blazer and Gratzek (1985) reported another case of proliferation in gill cartilage of fish infected with metacercariae of a digenean trematode. Bristow (1990) found immature final stage digeneans associated with mortalities in "postage stamp" larvae of Atlantic halibut *Hippoglossus hippoglossus* Linnaeus, 1758. To date, there is no chemotherapy to control metacercaria stage of digeneans in fish (Post, 1987). The impact of intestinal digeneans to fish has not been studied intensively, although such parasites are very common in most fish populations.

Environmental effects of many parasitic diseases are well recognized (Liang *et al*., 2007) and parasites have been used as markers of biosystem health and stress (Marcogliese, 2005; Dzikowski, Paperna and Diamant, 2003). Landsberg *et al.* (1998) showed that parasites of fish are more sensitive to environmental stress than are the fish themselves. These authors described the response of specific parasites to particular environmental factors, for example, the response of monogeneans to temperature, nematodes to contaminants and protists to low dissolved oxygen. The parasite community may also be affected by the presence and/or relative abundance of benthic macro-invertebrates, many of which are intermediate hosts in the digenean lifecycle (Möller and Anders, 1986). In this study, fish in ponds and cages are living in two different environmental systems. The fish density in ponds was much lower than that in cages, the depth of the cage was usually 3-4 meters, while that of the ponds was 1.0-1.5 meters, the surface area of the ponds was much larger than that of the cages. These differences may contribute to different parasite burdens of *P. pacificus* on cage- and pond-reared *E. coioides*. Other than *P. pacificus*, there was no difference in the prevalence of infection of the other parasites between the two culture systems; this may be due to the relatively small sample size. The life cycles of these parasites are complicated; the prevalence of infection may be affected by the biology of the intermediate host rather than environmental factors.

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Parasitic caligid copepods of farmed marine fishes in the Philippines

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ABSTRACT

Recently, heavy infestation of caligid copepods occurred among farmed rabbitfish *Siganus guttatus*, pompano *Trachinotus blochii* and sea bass *Lates calcarifer* in the Philippines. In *S. guttatus* broodstock, *Caligus epidemicus*, *Pseudocaligus uniartus* and *Lepeophtheirus sigani* concurrently caused severe erosion and hemorrhaging of the body surface, fins and eyes of affected fish occurring at 95.78%, 1.52% and 0.70% of the parasite load, respectively, and with associated mortality of the host fish. In marketable-sized *T. blochii*, *L. spinifer* caused body lesions that considerably reduced the market value of harvested fish. In *L. calcarifer* juveniles, infestation with *C. epidemicus* resulted to loss of appetite, lethargy and stunted growth of affected fish. Because of its pathogenicity, low host specificity and tolerance to brackish water, *C. epidemicus* poses the highest threat to farmed marine fish in the Philippines. *Lates calcarifer* and *T. blochii* are new host records for *C. epidemicus* and *L. spinifer*, respectively. This is also the first record of *L. spinifer* in the Philippines.

Key words: caligid, rabbitfish, pompano, sea bass, sea lice

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INTRODUCTION

Marine fishes including rabbitfish (*Siganus guttatus*), pompano (*Trachinotus blochii*) and sea bass (*Lates calcarifer*) are widely cultured in Southeast Asian countries. In the Philippines, the nursery and grow-out phases of culture of marine fishes are conducted in floating net cages or earthen ponds. With the intensification of the aquaculture industry in the region, heavy losses due to diseases including viral, bacterial and parasitic infections have become one of the current major concerns (Leong, 1998; APEC/SEAFDEC, 2001; Ho *et al*., 2004; Moravec, Cruz-Lacierda and Nagasawa, 2004; Nagasawa and Cruz-Lacierda, 2004).

One parasitic problem consistently observed in marine and brackish water fish culture throughout the world is the infestation with copepods belonging to family Caligidae or commonly known as sea lice. The impacts of sea lice infection in farmed marine fishes have been recently reviewed, with reported disease outbreaks and high mortalities (Johnson *et al*., 2004; Lester and Hayward, 2006). The problem of parasitic copepods as disease causing agent has become not only more frequent but also more intensive. In this paper, we report three disease cases associated with caligid infestation on cultured *S. guttatus*, *T. blochii* and *L. calcarifer* in the Philippines.

MATERIALS AND METHODS

Siganus guttatus. Recently, an extremely heavy infestation of caligid copepods with associated mortality occurred among tank-reared broodstock of rabbitfish *S. guttatus* at the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Iloilo, Philippines. The fish were being used for nutritional requirement studies when the infestation occurred. Twenty five fish each (total length [TL] = 26-29 (mean 28) cm; body weight [BW] = 250-500 (mean 400) g) were reared in two 10-tonne circular, canvas tanks. The fish were a mixture of wild-caught and hatchery-reared broodstock. Fish were fed twice daily at 3% of body weight with formulated diet and supplied with flow through sand-filtered water and aeration. Water temperature and salinity ranges recorded during the experiment were 27-29°C and 30-32 ppt, respectively. Five months into the experiment, the experimental fish developed severe erosion and hemorrhaging of the body surface with associated mortality. Two severely affected fish were sacrificed, body measurements were taken, and examined for external and internal parasites. Live caligids were observed microscopically and measurements were taken. The rest of the caligids were fixed in 70% ethanol. The remaining infected rabbitfish broodstocks were subjected to flow through freshwater for four hrs as an immediate control measure.

Trachinotus blochii. Marketable-sized cage-cultured pompano *T. blochii* exhibited disease signs such as loss of appetite, weakening, thinning and with areas of scale loss on the body surface. The affected fish (mean BW=350 g, n=10) had been cultured for one year, prior to the observation of disease signs, in 5 x 5 x 5 m floating net cages in a commercial farm located in Morong, Bataan, west of Bataan province in Central Luzon, Philippines.

Fish were fed twice daily at 1.5-5% of body weight with a commercial diet. Affected stocks showing disease signs were immediately harvested and iced prior to marketing. During this time, iced fish exhibited obvious hemorrhagic lesions on the body surface, with heavy infestation of parasites on affected areas. The parasites were collected, preserved in 10% formalin and submitted to one of the authors (E.R. Cruz-Lacierda) for identification. Water temperature and salinity prior to fish harvest were 29-30°C and 30 ppt, respectively. The remaining stocks, upon our recommendation, were bathed in freshwater for 15 min as an immediate control measure. Two weeks after the incidence, ten fish were brought to the laboratory and examined for the presence of caligids. Collected caligids were preserved in 70% ethanol and examined under the microscope for morphological study.

Lates calcarifer. Sea bass *L. calcarifer,* cultured in 3 x 2 x 1.3 m floating net cages installed inside an earthen pond at Dumangas Brackishwater Station of SEAFDEC AQD, with stocking density of 40 fish per sq. m., exhibited disease signs such as loss of appetite, weakening and stunted growth during an experiment on formulated diets for grow-out culture of *L. calcarifer* in ponds. Affected fish (TL=8.6-11.7 (mean 10.4) cm; BW=8-13 (mean 10.9) g, n=10) were collected, transported live to the laboratory and examined for external and internal parasites.

RESULTS

Siganus guttatus. The fish showed severe erosion and hemorrhaging of the skin on the ventral side of the body, particularly below and between the pectoral and pelvic fins (Fig. 1). The eyes and fins also show hemorrhagic lesions. A 10% mortality was recorded among the affected stock. Continuous heavy rains that made incoming water turbid were also noted a few days before manifestation of disease signs.

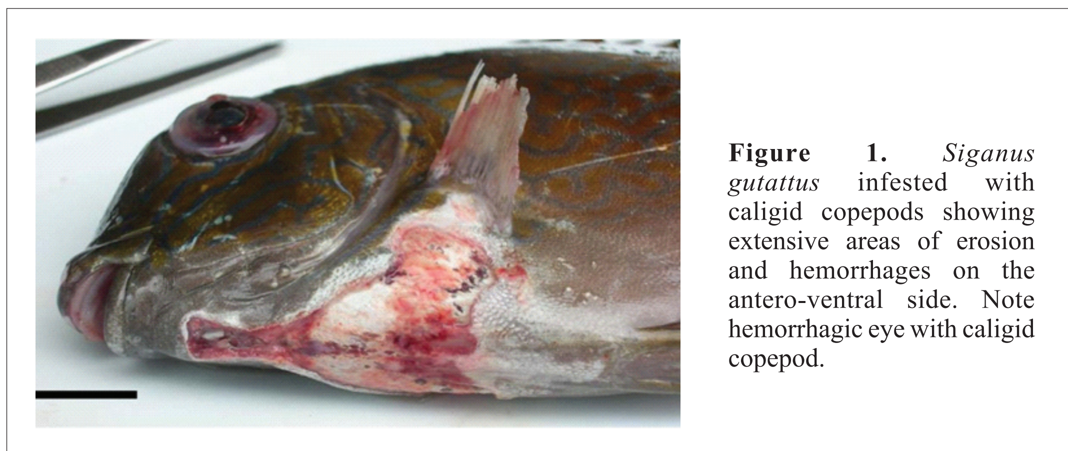

Figure 1. *Siganus gutattus* infested with caligid copepods showing extensive areas of erosion and hemorrhages on the antero-ventral side. Note hemorrhagic eye with caligid copepod.

The affected fish showed heavy infestation of caligid copepods. They were attached to the body surface (Fig. 2), eyes and fins of the host fish. Further examination of the collected parasites revealed three species of caligids, *Caligus epidemicus* Hewitt, 1971, *Pseudocaligus uniartus* Ho, Kim, Cruz-Lacierda and Nagasawa, 2004 and *Lepeophtheirus sigani* Ho, Kim,

Figure 2. *Siganus gutattus* with caligid copepods on body surface.

Cruz-Lacierda and Nagasawa, 2004, with *C. epidemicus* occurring at 95.78% of the parasite load (Table 1). Most of the *C. epidemicus* were pre-adult, adult and ovigerous females. Adult females (n=20) and males (n=20) of *C. epidemicus* were 2.14 mm (range: 2.03-2.33) and 1.47 mm (range: 1.35-1.55) in mean body length (MBL), respectively. *Pseudocaligus uniartus* occurred at 1.52% and the adult females (n=4) and males (n=7) were 2.10 mm (range: 1.85-2.30) and 1.99 mm (range: 1.90-2.05) in MBL, respectively. A lower percentage of *L. sigani* occurred at 0.70% and the adult females (n=2) and adult males (n=4) were 2.37 mm (2.28-

Table 1
Intensity of caligid copepods on the body surface of rabbitfish *Siganus gutattus* broodstocks kept in land-based tanks of SEAFDEC AQD in Iloilo.

Fish no	TL (cm)	BW (g)	C. e.	P. u.	L. s.	Chal	Total
1	29	350	660	10	6	17	693
2	26	270	157	3	0	0	160

TL, total length; BW, body weight; *C. e., Caligus epidemicus; P. u., Pseudocaligus uniartus; L. s., Lepeophtheirus sigani;* Chal, chalimus larvae

2.45) and 1.58 mm (1.40-1.75) in MBL, respectively. A few chalimus larvae (1.99%) were also collected from the fins. As the majority of the caligids recovered were identified as *C. epidemicus*, we presume that the chalimus larvae recovered belong to *C. epidemicus*.

Examination of the internal organs revealed the camallanid nematode *Procamallanus guttatusi* (Andrade-Salas, Pineda-Lopez and Garcia-Magaña, 1994) in the stomach and intestine of one rabbitfish at an intensity of six and seven, respectively. No other external and internal parasites were observed.

The remaining infected stock when subjected to flow-through freshwater for four hrs dislodged and killed the caligids from the body surface of the rabbitfish. The fish when returned to full seawater with a salinity of 32 ppt recovered and no caligid infection was observed when fish were reexamined, thereafter.

Trachinotus blochii. Examination of the collected parasites (n=60) from the body surface of *T. blochii* revealed that they belong to family Caligidae and was identified as

Lepeophtheirus spinifer Kirtisinghe, 1937 by the fifth leg of females overreaching the caudal rami and carrying three plumose setae on the dorsal side (Pillai, 1985). The adult males (n=10) and females (n=10) of *L. spinifer* measured 2.73 (range: 2.40-2.88) and 4.24 (range: 4.05-4.40) mm in MBL, respectively. The affected fish carried approximately 50 copepods per fish (n=10) (J. Nealaga, pers. comm.). The harvested stocks when marketed have considerably reduced market value because of the lesions on the body surface (J. Nealaga, pers. comm.). The remaining infected *T. blochii* that were bathed in freshwater for 15 min and examined (n=10 fish) two weeks after the infection also carried *L. spinifer* with a prevalence of infection of 100% and mean intensity of infection of 3.7 (range: 1-5).

***Lates calcarifer*.** Examination of affected *L. calcarifer* (n=10) showed 100% prevalence of *C. epidemicus* with a mean intensity of 12.7 (range: 6-35). Although there was no mortality involved, the fish manifested disease signs such as loss of appetite and lethargy with reduced growth rate as the most obvious (E. Coniza, pers. comm.). Adult females (n=10) and males (n=10) of *C. epidemicus* were 2.10 (range: 1.88-2.28) and 1.50 (range: 1.38-1.55) mm in MBL, respectively. No other parasite was recovered in all examined fish.

DISCUSSION

Parasitic copepods belonging to family Caligidae, also known as sea lice, with those belonging to two genera *Caligus* and *Lepeophtheirus* are the most commonly reported species on marine and brackish water cultured and wild marine fish (Ho, 2000, 2004; Johnson *et al*., 2004). In the Asia-Pacific region, many caligid species have been reported to affect growth and survival of their hosts (e.g. Fujita, Yoda and Ugajin, 1968; Hewitt, 1971; Laviña, 1978; Jones, 1980; Lin and Ho, 1993; Lin, Ho and Chen, 1996b), especially in intensive aquaculture systems.

Caligus epidemicus has been reported to be widely distributed and caused mass mortality in several species of fish in Australia (Hewitt, 1971) and in tilapia in Taiwan (Lin and Ho, 1993; Lin, Ho and Chen, 1996a). In the Philippines, *C. epidemicus* has been recorded on at least 12 host fishes, with a high prevalence rate and at high intensity (Natividad, Bondad-Reantaso and Arthur, 1986; Ho *et al*., 2004). However, *L. calcarifer* has not been previously recorded as host for *C. epidemicus*. Thus, *L. calacarifer* is a new host record for *C. epidemicus*.

Among the caligids reported here, *C. epidemicus* poses the highest threat to marine aquaculture in Asian waters because of its pathogenicity, low host specificity and tolerance to brackish water (Hewitt, 1971; Ho, 2000, 2004; Ho *et al*., 2004). The other caligids, *P. uniartus* and *L. sigani* have been reported to be host-specific to rabbitfishes (Ho *et al*., 2004) while *L. spinifer* so far has been recorded from *Chlorinemus* sp. in Sri Lanka (Kirtisinghe, 1937) and from *C. lysan*, *C. tala* and *Rachycentron canadus* in India

(Pillai, 1985). *Trachinotus blochii* is a new host record for *L. spinifer*. The checklist of parasites of fishes from the Philippines (Arthur and Lumanlan-Mayo, 1997) shows that this is also the first record of *L. spinifer* in the country.

The morphology of *C. epidemicus* has been previously described (Ho and Lin, 2004) as well as *P. uniartus* and *L. sigani* (Ho *et al*., 2004). The morphology of *L. spinifer* will be re-described in a separate paper.

In the present study, majority of the *C. epidemicus* were pre-adult, adult and ovigerous stages, with chalimus larvae comprising only two percent of the caligid population. It has been reported in previous studies that the highest proportion of pre-adult and adult *Lepeophtheirus salmonis* in sea trout (*Salmo trutta*) coincided with the lowest prevalence of chalimus larvae (Tully *et al*., 1993; Tingley, Ives and Russell, 1997; Schram *et al*., 1998) and that such pattern is an indication of low transmission rate of the parasite.

Although severe erosion of the skin was confined on the ventral portion of the head, many copepods were also observed on the whole body surface, fins and eyes of the rabbitfish, similar to *C. epidemicus* infection on yellowfin bream, *Acanthopagrus australis* (Roubal, 1994). The lesions on the body surface of affected fish could be attributed to the feeding activity of the parasite (Kabata, 1974; Urawa and Kato, 1991). Further, similar disease signs and mortality caused by two caligids (*L. atypicus* and *C. oviceps*) were reported on another rabbitfish, *S. fuscescens*, cultured in Taiwan (Lin, Ho and Chen, 1996b). Parasitic copepods have been reported to feed on host's mucus, tissues and blood (Johnson *et al*., 2004).

It is highly possible that the wild-caught rabbitfish broodstocks used in the experiment may have had prior infection. The turbid incoming water brought about by continuous heavy rains may have also contributed to the outbreak of caligids in rabbitfish. The pompano and sea bass seed stocks, however, were hatchery-produced. Thus, caligid infections may have originated from the wild fish present in the vicinity of culture area and/or from other cultured fish species in the same culture site.

As an immediate control measure, bathing the remaining infected rabbitfish in flow-through freshwater for four hrs prevented further infection and mortality. The fish recovered (J.B. Gonzaga, pers. comm.) and no subsequent caligid infection was observed. The use of freshwater to control caligids and other marine parasites has been previously recorded (e.g. Urawa and Kato, 1991; Zafran *et al*., 1998, 2000; Cruz-Lacierda *et al*., 2004).

The use of chemicals such as formalin and hydrogen peroxide has been reported against caligids on groupers (Zafran *et al*., 1998, 2000; Koesharyani *et al*., 2001; Cruz-Lacierda and Pagador, 2004). However, control methods to prevent settlement, molting, growth and maturation of caligids should be looked into.

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Laboratory experimental infection of the freshwater snail *Gyraulus convexiusculus* (Hutton, 1849) and the bighead carp *Aristichthys nobilis* (Richardson, 1845) with the blood fluke *Sanguinicola armata* Plehn, 1905

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ABSTRACT

The route of cercarial penetration of *Sanguinicola armata* into the snail intermediate host *Gyraulus convexiusculus* and the fish definitive host *Aristichthys nobilis* was demonstrated through laboratory experimental infection experiments and histopathological studies. For snail infection experiments, fifty definitive host (*A. nobilis*) infected with the the blood trematode *Sanguinicola armata* were exposed to 5 naïve *G. convexiusculus* for 24 hours before snails were moved to another aerated aquarium which was filled with dechlorinated water and *Hydrilla* sp. Ten snails were sacrificed daily, their carapaces were removed from the tissues and wet smears were done and observed under compound microscope. This procedure was continued until day 17 for 17 replicates. Meanwhile, five snails were fixed in 10% formalin EDTA at days 5, 10, and 15 and snail tissue was processed for histopathology. For production and collection of cercariae, infected *G. convexiusculus* with *S. armata* were exposed to light to stimulate emergence of cercariae. Ten collected cercariae were introduced to one fish for 24 hours in 100 l fibreglass tanks. The same procedure was repeated for 240 fish. Three infected fish were examined daily until day 80. For penetration study of *S. armata* cercariae into *A. nobilis,* ten groups comprising ten naïve fishes were exposed to 300 cercariae at different time duration of 10, 20, 30, 40, 50 minutes and 1, 3, 6, 12 and 24 hours exposure. Each group was kept in 100 l fibreglass tanks. The fish from each group were divided into two. Some were cut vertically and some horizontally to get sections through all tissues. Then they were fixed in 10% buffered formalin for 48 hours and formalin EDTA for one week before histology studies. Results indicated that the majority of blood fluke adults were found in the bulbous arteriosus and histopathological results

Shaharom-Harrison, F., Seman, N.A. and Suhairi, M. 2011. Laboratory experimental infection of the freshwater snail *Gyraulus convexiusculus* (Hutton, 1849) and the bighead carp *Aristichthys nobilis* (Richardson, 1845) with the blood fluke *Sanguinicola armata* Plehn, 1905 pp. 63-72**.** *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.

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showed that adult *S. armata* caused increased thickness of gill arteries, while infection with eggs of the trematode caused hyperplasia of primary gill lamellae and filaments and secondary gill lamellae were fused resulting in chronic hyperplasia. This study on cercarial penetration of *A. nobilis* juveniles showed that the most abundant cercarial penetration into *A. nobilis* occurred at intervals of 30 minutes, 40 minutes and 180 minutes. Most penetration occurred at the caudal fin followed by abdominal and caudal peduncle, whereas least penetration site was the operculum.

Keywords: laboratory experiment, freshwater snail, bighead carp, blood fluke, *Sanguinicola armata*

INTRODUCTION

Studies on the life cycle of blood flukes have been studied by several authors (Meade and Pratt, 1965; Meade, 1967; Schell, 1974). Kua (1995) showed that *Gyraulus convexiusculus* is an intermediate host of the blood fluke *Sanguinicola armata.* Even though Kua (1995) did laboratory experimental infection of the snail, she did not show the route of penetration of the cercaria into the snail and grasscarp fingerlings. In this present, study histopathology studies on snails and fish subjected to laboratory experimental infections proved the route of cercarial penetration into both snail and bighead carp fingerlings as this blood fluke was also found to infect the bighead carp *Aristichthys nobilis.*

MATERIALS AND METHODS

1. Snail Infection Experiments

Laboratory experiment was carried out in 100 l fiberglass tanks to build up the source of infection, the blood trematode *S. armata* for infection of its intermediate host, the freshwater snail *G. convexiusculus* and its definitive host - the bighead carp *A. nobilis*. Each tank was filled with 50 l dechlorinated water and aerated. To provide the source of infection, 50 *A. nobilis* (size 2-3 cm) infected with *S. armata* and 5 naïve *G. convexiusculus* (which were kept in fine netlon -covered Petri dish) were placed in the same fiberglass tank. They were left inside for 24 hours before the *G. convexiusculus* were moved to another aquarium which was filled with dechlorinated water, *Hydrilla* sp. and aerated. Ten snails were sacrificed daily, their carapaces were removed from the tissues and wet smears were done. The slides were examined under compound microscope. This procedure was continued until day 17 for 17 replicates. Meanwhile, 5 snails were fixed in 10 % formalin EDTA at day 5, 10, and 15 and snail's tissue was processed for histological evaluation (Fig. 1).

2. Fish Infection Experiment

Ten *G. convexiusculus* (size 5 - 10 cm) infected with *S. armata* were put into 50 ml beaker (filled with 30 ml dechlorinated water) and exposed to the light source for approximately 30 minutes to stimulate the cercaria to abandon the snails. Water from beaker was poured into a cavity block and examined under a dissecting microscope to collect the cercariae using a

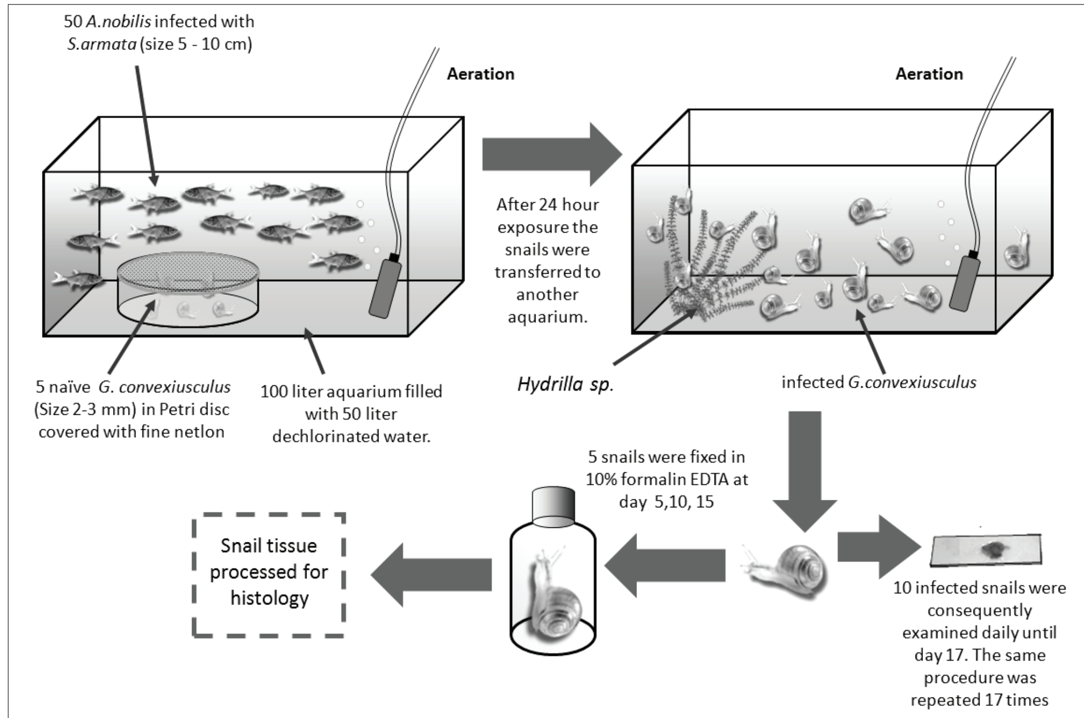

Figure 1. The illustration of methodology for snail infection experiments.

mounted needle. An individual naive fish (2 cm -3 cm) was introduced with 10 cercariae in the 50 ml beaker (filled with 30 ml dechlorinated water) in order to infect the fish for 24 hours. The same procedure was repeated with 240 fishes and they were kept together in one tank. Every day until day 80, 3 infected or exposed fish were sampled and examined (Fig. 2).

3. Penetration Study of *S. armata* cercaria into *A. nobilis*

Meanwhile, for penetration study of *S. armata* cercaria on *A. nobilis*, the above-mentioned light exposure method was used to collect the cercariae. Ten groups comprising 10 naïve fishes were exposed to 300 cercariae in 50 ml beaker (filled with 30 ml dechlorinated water). Each group was exposed to cercariae for a different time; for duration of 10, 20, 30, 40, 50 minutes and 1, 3, 6, 12, and 24-hours exposure. Each group was kept in 100 l fiberglass tank. The fish from each group were divided into two some were cut vertically and some horizontally to get sections through all tissues. Then they were fixed in 10 % buffered formalin for 48 hours and formalin EDTA for 1 week before histology studies (Fig. 3). Besides that, histopathology study was also focused on penetration of *S. armata* into the internal organs. of A. *nobilis* For the purpose of this study with ten *G. convexiusculus* (size 5 - 10 cm) infected with *S. armata* were put into 50 ml beaker (filled with 30 ml dechlorinated water) and were exposed to the light source for approximately 30 minutes to stimulate the cercaria to abandon the snails. Water from beaker was poured into cavity block

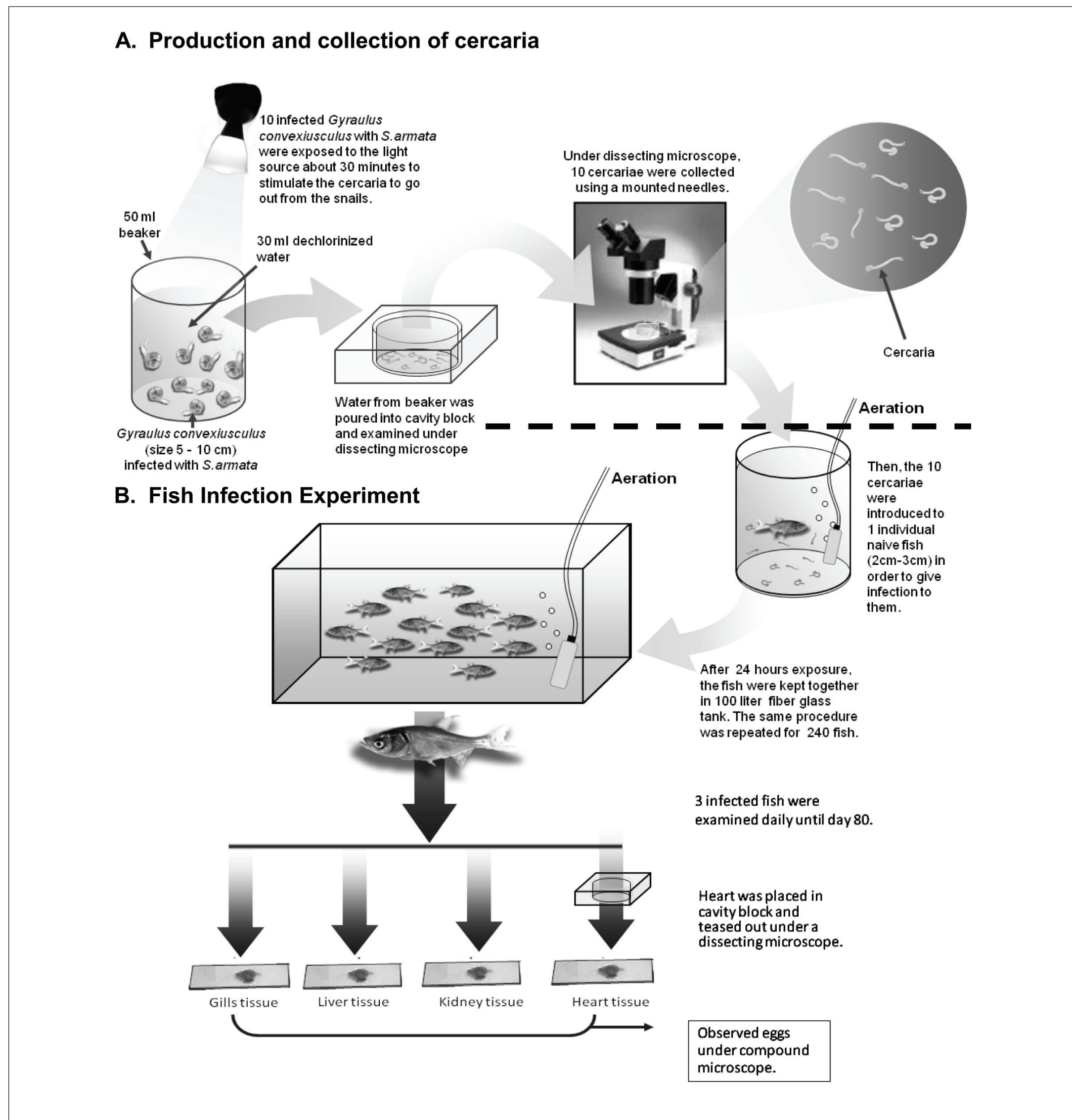

Figure 2. The illustration of methodology for fish infection experiment.

and examined under dissecting microscope to collect the cercariae using a mounted needle. With the intention to become infected an individual naïve fish (2-3 cm) was put into 50 ml beaker filled with 30 ml dechlorinated and addition of 10 cercariae. After 24 hours exposure, the fish was kept in 100 L fiberglass tank. Then after day 50, the fish was sacrificed and dissected and its' internal organs namely liver, kidney, heart and gills were cut separately and fixed in 10% buffered formalin for 48 hours before processing for further histological studies (Fig. 4).

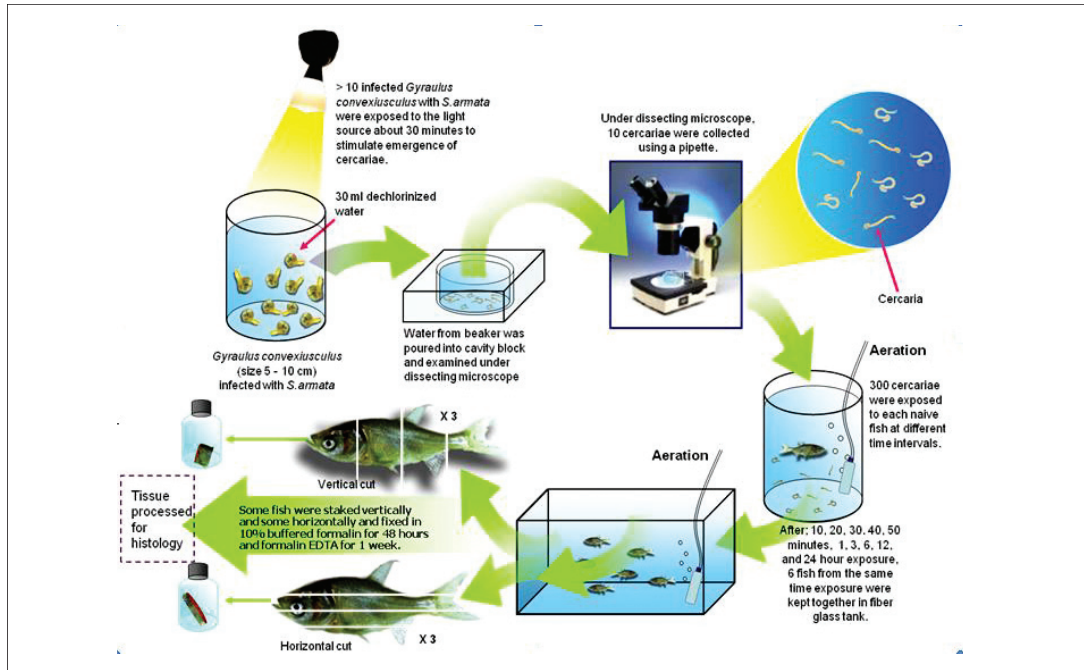

Figure 3. The illustration of methodology for histopathology study on penetration study of *S. armata* cercaria on *A. nobilis*.

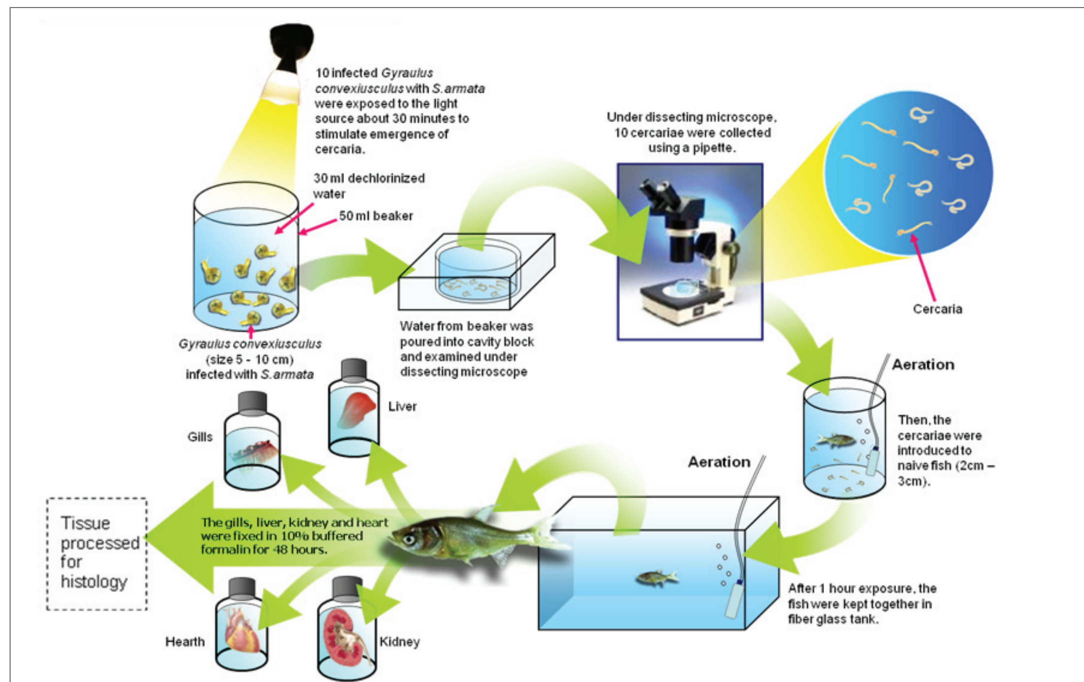

Figure 4. The illustration of methodology for histopathology study on the infection of *S. armata* on *A. nobilis*.

RESULTS AND DISCUSSION

The results of our experiments show that majority of blood fluke adults were found in the bulbous arteriosus and is in accordance with the findings of Kua (1995). Results of histopathological examinations showed that adult *S. armata* caused increased thickness of gill arches (Fig. 5.ii). Infection with eggs of *S. armata* causes hyperplasia of primary gill lamella, filaments and secondary gill lamellae were fused resulting in chronic hyperplasia (Fig. 5.iii – 5.v). This finding is in compliance with similar findings described by Evans (1974); Ogawa *et al.* (1989) and Padros *et al*. (2001). Encysted eggs in the gills filaments were surrounded by fibrotic tissue (Fig. 5.vi). The majority of the eggs were found in the gills, followed by liver, kidney and heart ventricle (Fig. 6). Maximum-sized eggs were found in the gills, kidney, liver and heart ventricles (Table 1). The study on cercarial penetration to *A. nobilis* juveniles showed that the most abundant cercarial penetration into

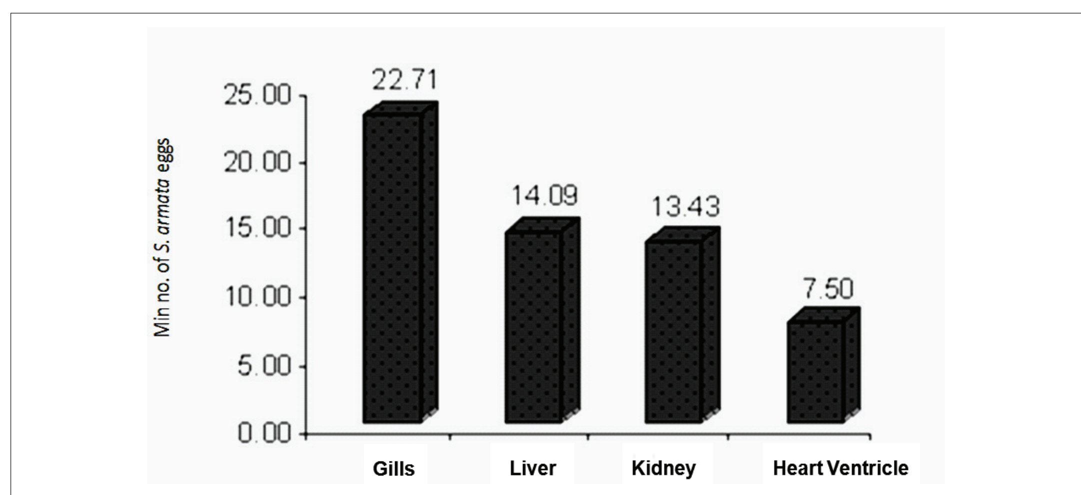

Figure 6. Mean number of *S. armata* eggs vs the body parts of *A. nobilis.*

Table 1. The size of *S. armata* eggs (n= 228) from infection study of *S. armata* on *A. nobilis.*

	Length (µm)	Width (µm)
	(Mean ± SD)	(Mean ± SD)
Gills	38.93 ± 6.77	19.09 ± 1.39
Kidney	38.11 ± 7.50	18.35 ± 1.53
Liver	37.50 ± 6.26	18.11 ± 1.60
Heart	37.38 ± 7.53	18.05 ± 1.33

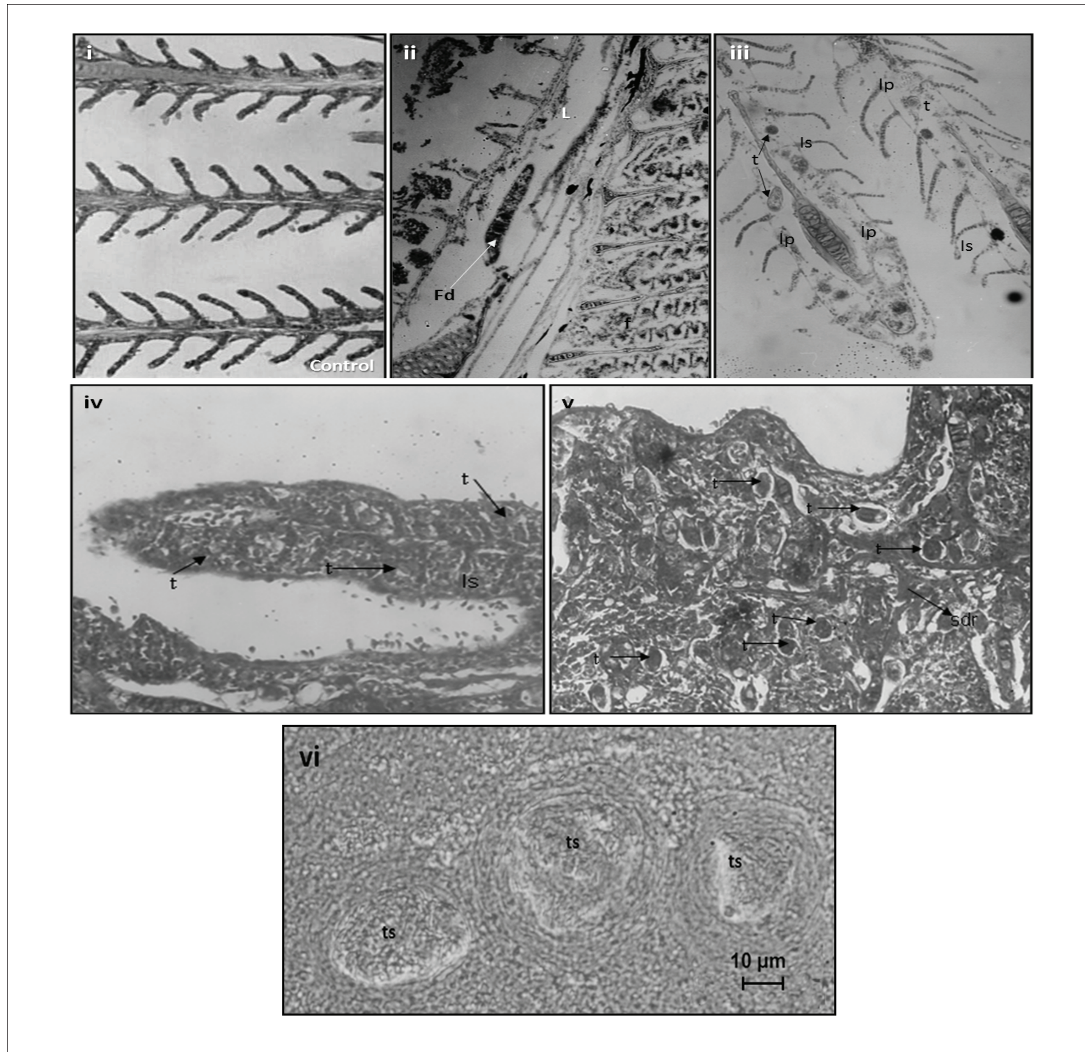

Figure 5. Histological changes in gills. Figure 5.i. Normal histological appearance of the gills from control fish. H&E stain, 10 x magnifications. Figure 6.ii. The infection with adult *S. armata* (Fd) cause the dilation of branchial artery of *A. nobilis*. f = gill filaments, L = branchial artery. H&E stain, 10 x magnifications. Figure 6.iii. The infection with *S. armata* eggs cause hyperplasia of secondary lamella (lp), t = eggs of *S. armata*, ls = secondary lamella, tr = cartilage. H&E stain, 20 x magnifications. Figure 5.iv. Heavy infections with *S. armata* eggs (t) cause fusion of secondary gill lamella (ls) and forming chronic hyperplasia. H&E stain, 100 x magnifications. Figure 5.v. Heavy infections with *S. armata* eggs on *A. nobilis* gills cause fusion of secondary lamellae and chronic hyperplasia. sdr = blood vessel. H&E stain, 20x magnification. Figure 5.vi. The encysted eggs isolated from the gills filaments were surrounded by fibrotic tissue forming granuloma (wet mount).

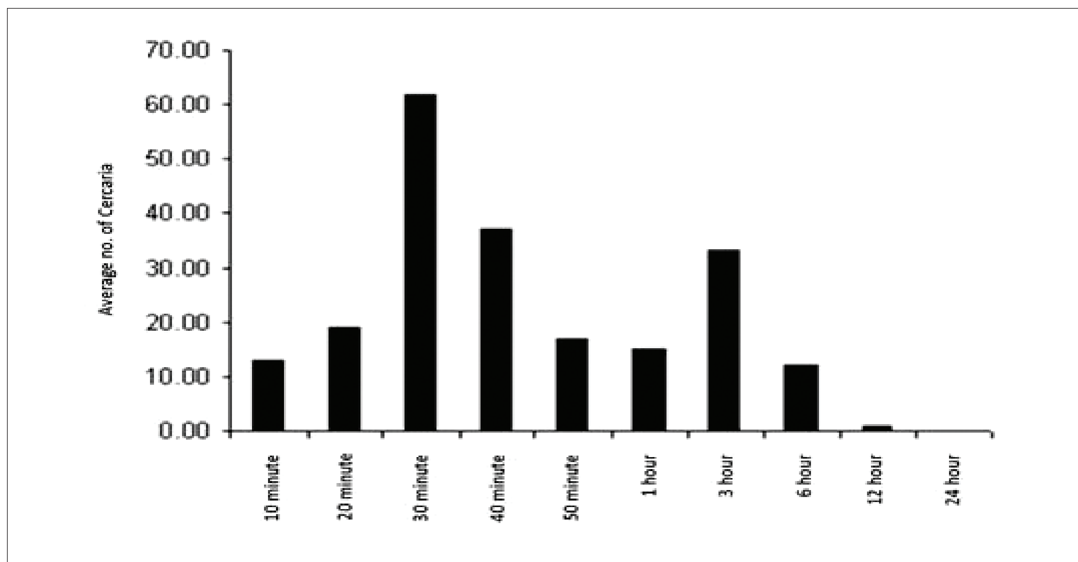

Figure 7. Number of cercaria vs. time exposure

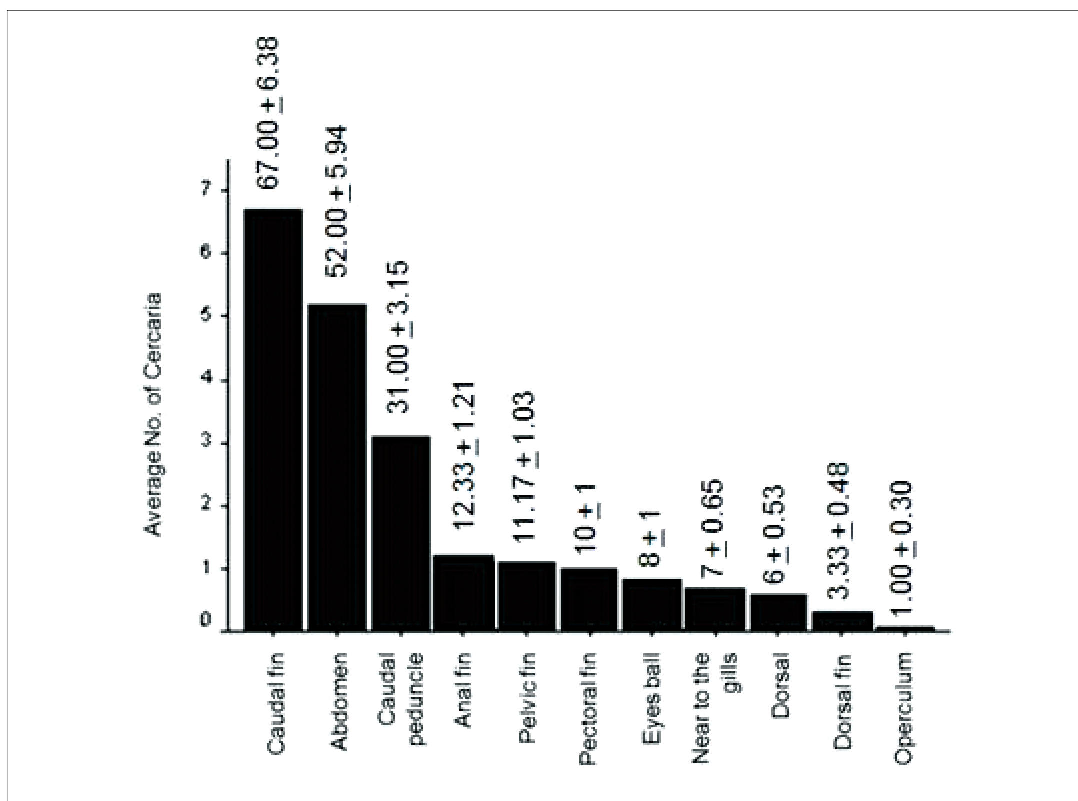

Figure 8. Abundance of cercaria in different sites of penetration.

A. nobilis occurred at intervals of 30 minutes, 40 minutes and 180 minutes (Fig. 8) similar to the findings of Maede and Pratt (1965); Maede (1967); Schell (1974), Sommerville and Iqbal (1991). The part of penetration was the caudal fin, followed by abdominal and caudal peduncle, whereas least penetration site was the operculum (Fig. 8).

CONCLUSIONS

Experimental infection of snails with blood fluke and the histopathological research of its route of penetration into the body of bighead carp *A. nobilis* gave us the established evidences that freshwater snail *G. convexiusculus* is the intermediate host of the digenetic blood trematode *S. armata*. Results of our study confirmed the presence and histopathological lesions in gills, heart, kidney and liver of the host.

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Current trends in the study of molluscan diseases

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ABSTRACT

The study of molluscan diseases has a long history. The first publication on the redial stages of a trematode appeared in the 18th century; early papers on molluscan phagocytosis appeared in the last half of the 19th century and yet much work published before about 1975 does not appear in electronic abstract databases and is effectively "lost". By contrast, a recent search of a leading abstract database for the terms "mollusc" and "disease" shows that the number of publications has exploded in the last eight years and the exponential trend looks set to continue. Much of the increase has been driven by the introduction of molecular technologies, the rediscovery that the immunology of invertebrates generally is a rich hunting ground for new biochemical defence systems and thus potential medical breakthroughs and the desire to publish multiple papers from the same project. As this publication trend continues, it will become increasingly difficult to be knowledgeable on all aspects of molluscan diseases and considerable specialisation is inevitable.

It is not only our knowledge about known mollusc diseases that has grown, since new diseases continue to be reported as: aquaculture becomes more intensive; the Asia/Pacific regional skills base develops; and international reporting becomes more accurate. Transfer of disease between jurisdictions is also becoming more rapid as products are sent live around the world both as broodstock and for human consumption. Thus, the work of the Network of Aquaculture Centres in the Asia and the Pacific and the Food and Agriculture Organization of the United Nations in awareness raising and skills development will continue to make an impact. It is inevitable that, as the initial work on mollusc diseases developed around shellfish growing areas in Europe and America, the next generation of molluscan disease experts will be based in the Asia and the Pacific region.

Key words: diagnosis, taxonomy, physiology, parasite-host relationships

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INTRODUCTION

Global demand for seafood, including molluscs, continues to grow (FAO, 2009). However, disease continues to be a major financial constraint to growth of mollusc culture. Losses due to *Marteilia refringens* and *Bonamia ostreae* in French oyster farms over the period 1980-1983 were estimated at US$31 million dollars (Grizel and Héral, 1991). Abalone mortalities of unknown aetiology in Taiwan cost US$11 million (Bondad-Reantaso and Subasinghe, 2005). Ongoing mortalities in abalone in Australia had, by February 2007, resulted in a loss of US$4.5 million in exports (Lannen, 2007).

The study of molluscan diseases has a long history. The first publication on the redial stages of a trematode appeared in the 18th century (Swammerdam, 1737), based on work he completed during the 17th century. Early papers on molluscan phagocytosis appeared in the last half of the 19th century (Metchnikoff, 1893; De Bruyne, 1893, 1896) as well as early papers on the histology of molluscan hosts (Grey, 1853; Peck, 1877). According to Yonge (1926), in his excellent description of the histology of the digestive diverticula in lamellibranchs, it was in 1880 that the name “hepatopancreas” for the digestive gland of crustacea was first used, a term still sometimes used for the digestive diverticula in molluscs. Unfortunately, much work published before about 1975 does not appear in electronic abstract databases and is effectively “lost”.

By contrast, a recent search of CABI® abstract database for the terms “mollusc” and “disease” showed that the total number of publications in the database for the period 1900-1950 was only 16, all of which were human or veterinary health references, and clearly missed all of the pertinent aquatic mollusc disease papers. In the next 25 years (1951-1975) there were 55 references, only three of which were on aquatic molluscs and none of which included those cited above. The next 25-year period (1976-2000) revealed 1 815 references, yet in the next eight years there were 10 814 references, of which 1 174 were of aquatic relevance. A similar trend is evident when using Aquatic Sciences and Fisheries Abstracts (ASFA) or other such databases. While clearly, much work (including foreign language papers) is not being captured by such databases, it is clear that this exponential trend is set to continue. It also means that, where it was possible to have read all of the literature on the subject up to about 1990, it is now no longer the case. As this publication trend continues, it will become increasingly difficult to be knowledgeable on all aspects of molluscan diseases and considerable specialisation in a team environment is inevitable (Sparks, 2005; Whitfield, 2008).

The increasing specialisation has been driven by a number of factors including the:

- introduction of molecular, genomic and proteomic technologies; microfluidics and the development of microfluidic biochips capable of continuous sampling and real-time (and remote) testing of air/water samples for pathogens and toxins;
- rediscovery that the immunology of invertebrates generally is a rich hunting ground for new biochemical defence systems and thus potential medical breakthroughs;

- science-wide moves to publish frequently, which forces scientists to publish their research in parts, rather than waiting until the research has been completed. In this regard there is a clear trend towards papers with a multitude of authors; and
- increase in number of scientists in general.

For the purpose of this review, the current trends in the study of mollusc diseases can be divided into three categories: (i) diagnostic testing for known mollusc diseases; (ii) taxonomic and phylogenetic studies on pathogens and their host molluscs; and (iii) investigations of known diseases, their impact on the host and the environment. Of necessity, these categories are artificial – the boundaries between them tend to merge.

(i) Diagnostic testing of molluscs for disease.
Diagnostic tests fall into three broad categories:

- Screening apparently healthy animals for specific pathogens of concern. This is most commonly applied to stocks destined for live transfer or as part of a surveillance program.
- Determining the cause of poor health/mortality. This can often be a very complex process in determining the relationships between the host, pathogens (there may be more than one) and the environment (Snieszko, 1974; Berthe, 2002; Garnier *et al.*, 2007).
- Development of new test methodologies or procedures, or improvement of existing procedures. This is becoming more important as issues of sensitivity, specificity and fitness for purpose become more important.

Unfortunately, the results derived from molecular methods are sometimes at odds with more conventional methods, but too often the assumption is made that a positive polymerase chain reaction (PCR) result verifies an infection in a tested host, or that a negative PCR means that infection is not present. This assumption is valid only if the assay has been properly validated for the geographic area and for the hosts examined (Burreson, 2008). For example, based on histology, epidemiology and visualised by Transmission Electron Microscopy (TEM), Hine, Wesney and Hay (1992) and Hine, Wesney and Besant (1998) reported the presence of a herpes virus in oysters (*Ostrea chiliensis* and *Crassostrea gigas*) in New Zealand, which is certainly there (Jones, unpublished TEM obs.). A more recent study using histology and confirmatory PCR (Webb, Fidler and Renault, 2007) failed to amplify ostreid herpesvirus (OsHV-1) from New Zealand shellfish, leading the authors to discount the previous observations and question whether OsHV-1 is present in New Zealand at all. Ulrich *et al.* (2007) claim, based only on PCR results, the presence of *Haplosporidium nelsoni* infections in the Gulf of Mexico despite the results of thousands of oysters having been examined by histology from the same area for over 20 years which failed to observe a single infection (Burreson, 2008). PCR may show that parasite DNA[1] is apparently present in a sample, but in the absence of independent verification it does not confirm or refute infection in the environment (see also Kanagawa, 2003).

(ii) Taxonomic and phylogenetic studies on pathogens and their host molluscs

Work to classify the large numbers of pathogenic organisms associated with molluscs has suffered from the ongoing decline in numbers of trained taxonomists. It is becoming difficult to find people who can identify and describe new metazoan parasites, especially since all of the classical taxonomic literature is based on morphology, and relatively few species are represented in Genbank. Nevertheless, accurate identification of both the host and the parasite is of importance. For example, Nakano and Spencer (2007) used phylogenetic analysis of DNA sequences to show that a species of small intertidal limpet *Notoacmea helmsi* from New Zealand was in fact a taxon comprising 5 morphologically cryptic species. Any study of the parasitology of the group would have been confounded by this discovery.

The current taxonomic difficulties are well illustrated by studies of the molluscan parasites informally grouped as "microcells" because of their small size (about 2 microns). The genera *Marteilia, Mikrocytos*, *Bonamia* and *Haplosporidium* spp. are relatively easily detected by routine histology, but species determination is much more problematic. *Marteilia refringens* and *Marteilia maurini* are morphologically indistinguishable (Longshaw *et al*., 2001) but can be separated by molecular means (Le Roux *et al*., 2001) and both infect oysters and mussels. López-Flores *et al*. (2004) suggested that the *Marteilia* from oysters and from mussels may be two different strains of the same species that appear to readily infect both hosts while other authors accept that they are closely related species - thus their taxonomic status is still open to debate (Berthe *et al*., 2004). A similar situation occurs with the occurrence of *Bonamia* sp. in Australia that has molecular and histological differences to *B. exitiosa* (J. Handlinger, pers. comm.). The distinction is important, for some species are internationally reportable, while other species of the genus are not.

While the use of sequence data from the small subunit ribosomal RNA (SSU r RNA[2]) gene is now commonly referred to in the description of new haplosporidians (Azevedo *et al*., 2006), sequence data alone is generally not sufficient to separate a new species. The distance between *Bonamia* sp. from New South Wales (Australia) and *B. exitiosa* from New Zealand over the 1 586 base pair sequence from the 18S gene is only 0.9% (Corbeil *et al*., 2006), yet there are differences in geography, morphology, ultrastructure and histopathology between the two microcells (B. Jones pers. obs., J. Handlinger, pers. comm.)

(iii) Investigations of known diseases of molluscs, their interaction with the host and the environment

There is a rich and growing body of literature on molluscan diseases and their associated pathogens, their interactions with each other and with the wider environment. Comparative genomics is also a source for major advances in our understanding of the regulatory systems not only in molluscs but also in their parasites.

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[1] Deoxyribonucleic acid

[2] Ribonucleic acid

In order to make some sense of the voluminous literature, it is useful to adapt Figure 1 below, developed by Snieszko (1974), to show the interrelationships of the host, parasite and the environment and to categorise the types of studies undertaken, as follows:

- **Host-parasite interactions** fall into two groups: firstly, the investigation of molluscan host impact on the pathogen (including host defence mechanisms involving the detection of and subsequent neutralising of pathogen); and secondly, studies on the impact of the parasites on molluscan host (including mechanisms by which the parasite overcomes the host defences and appropriates the host to its own purposes).
- **Parasite-environment interactions** can also be divided into two groups: the investigation of the pathogens impact on environment and the investigation of the environments effect on the pathogen (as opposed to the environmental impact on the host).
- **Environment-host interactions** include investigation of the environment on the host mollusc (including the effects of stress, pollution, tumours- environmental diseases); and investigations of how the host mollusc alters or affects the environment.

Host-parasite interactions

a) Impact of host on pathogen

Host defense mechanisms now constitute a rich area of research. By the end of last century, it was thought that molluscs did not have an acquired immune system and lacked immunoglobulin antibodies (Chu, 1988). Defense was ascribed to both cellular and humoral factors with phagocytosis as the primary response to foreign matter (Feng, 1988).

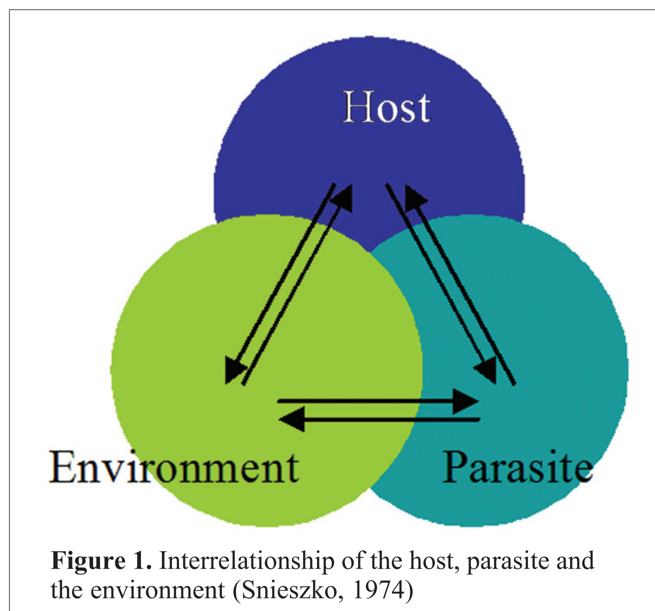

Figure 1. Interrelationship of the host, parasite and the environment (Snieszko, 1974)

Phagocytosis, in particular was studied in the 1890's and that early work was built on by Stauber (1950) who studied the effect of injected India ink particles in *Crassostrea virginica*. There is clearly variation in haemocytes among molluscs; for example, scallops and abalone do not have granulocytes yet other gastropods do (Hine, 1999; Travers *et al.,* 2008; Mahilini and Rajendran, 2008). Despite over 100 years of research and numerous papers describing the various morphological forms that haemocytes display, Allam, Ashton-Alcox and Ford (2002) were still able to write "*the origin, life cycle and life span of bivalve haemocytes are still largely unknown and the role of each cell type has not been completely elucidated*". Harris, Lambkin and O'Byrne-Ring (2006) and others have been using immunohistochemistry to identify structural and functional proteins in abalone leading Travers *et al*. (2007) to recommend that resolving the controversies over the classification of haemocytes in molluscs would require the "obligatory" use of mollusc-specific antibodies and gene probes. Whether the application of these new technologies will settle the controversy remains to be seen. Part of the problem is probably an assumption that haemocyte form and function will be the same across all molluscan groups, and a review, such as that by Hine, Wain and Boustead (1987) for teleost leucocytes, is well overdue.

It could have been added that both the origin and fate of haemocytes is also unknown. Haemocytes can be seen to pass through the intact columnar epithelial layers, especially those of the gut, in a process known as diapedesis (from the Greek '*diapedan*' = to ooze through). Though in human pathology the term is confined to the passage of blood cells through unruptured vessel walls, in invertebrates, especially molluscan pathology, the term is also used for the passage of haemocytes, which may or may not contain phagocytosed material, across epithelial borders to the exterior of the body (Onstad *et al*., 2006). Cheng (1967) noted that it was unknown if haemolymph was lost during diapedesis and what factors influenced the rate of diapedesis. That is still unknown and it is also still unclear what role diapedesis plays in the response to infectious diseases.

What we do know is that haemocytes are both mediated by and also produce "humoral factors". For example, killing mechanisms associated with haemocytes involve reactive oxygen molecules, such as the phenoloxidase cascade, that are now known to be important for phagocytosis, melanisation and encapsulation. Inducible serum antimicrobial factors including lysozyme (a bacteriolytic protein) are released by degranulation when phagocytosis occurs (Cheng *et al.*, 1975; Mohandas, Cheng and Cheng, 1985; Xu-Tao Hong, Li-Xin Xiang and Jian-Zhong Shao, 2006). However, the distinction between immunomediators, hormones and neurotransmitters, has become blurred by the finding that haemocytes can synthesis neuroendocrine peptide hormones and also have receptors for these peptides (Ottaviani and Franceschi, 1998a, 1998b).

Though most work has concentrated on the haemocyte-mediated response, other defense mechanisms have been studied. Wright (1959) demonstrated species specific substances in the mucous of a number of snail species and Cheng, Shuster and Anderson (1966a, 1966b) showed that haemolymph of *C. virginica* and *C. gigas* will stimulate cercariae of *Himasthla quitessetensis* to encyst, immobilising them and preventing infection.

There has been increasing activity looking for bioactive compounds in molluscs. β-glucuronidase, phosphatases, lipases, aminopeptidase amylase and antimicrobial factors have been described from molluscs (Chu, 1988; Montes, Durfort and Garcia-Valero, 1996; Montes *et al*., 1997; Roch *et al*., 2008). Agglutinins including haemaglutinins have also been widely reported and these may increase phagocytosis by acting as opsonins (Olafsen *et al*., 1992). Faisal, Oliver and Kaattari (1999) also showed that resistance to *Perkinsus* infections by *Crassostrea* species is effected by protease inhibitors to the extracellular serine proteases secreted by the parasite.

The finding of virus particles in molluscs, when looking for causes of disease, is complicated by the ability of molluscs to sequester live viruses (such as norwalk and hepatitis viruses) from their environment. The reason why this occurs and the relationship of these sequestered viruses to the host mollusc immune systems are unknown. The virus is not simply bioaccumulated since recent work (Le Guyader *et al*., 2006; Tian *et al*., 2007) suggests that clams, mussels and oysters trap the norwalk virus (or the virus actively binds) through an intestinal type A-like histo-blood group antigen. Flegel proposed in 1998 that crustaceans accommodate live virus as a means of continually challenging the immune system in the absence of an acquired immune system (Flegel, 2006). Berthe (2002) also suggested that the invertebrate immune system response is complex and suggests that an 'ecological', or whole system approach to immunology should be considered, rather than relying on the mechanistic 'cause-effect' interpretation of host-pathogen relationships that have, to date, dominated studies on infectious diseases of invertebrates.

b) Impact of pathogen on host

It has long been hypothesised that pathogens may be able to secrete extracellular products to inhibit the host response. For example, there is a strong negative association between the presence of *Marteilia sydneyi* and phenoloxidase activity in *Saccostrea glomerata*, possibly through the release of serum proteases, as happens with *Perkinsus marinus* (Faisal *et al*., 1999; Peters and Raftos, 2003). Variations in phenoloxidase also affect the resistance of oysters to QX disease[3] (Peters and Raftos, 2003; Bezemer *et al.,* 2006; Aladaileh, Nair and Raftos, 2007). Bezemer *et al*., (2006) used native-PAGE[4] to identify five discrete forms of phenoloxidase in wild oysters, of which one was associated with disease susceptibility. This raises the question, is it the lack of a specific phenoloxidase type that permits disease or can the pathogen "knock out" a specific phenoloxidase molecule?

Some pathogens are clearly able to inhibit or modify the host response. For example, *Bonamia roughleyi* microcells stimulate phagocytosis by suitable haemocytes but are not killed and instead proliferate within the host cell, eventually lysing the host to release more microcells (Da Silva *et al.,* 2008). The cycle results in massive destruction of haemocytes leading to death of the host oyster.

[3] QX stands for Queensland Unknown the title given to this disease prior to the discovery of the organism that is now known to cause it.

[4] Native-Page stands for native polyacrylamide gel electrophoresis

Parasites may also affect biochemical processes other than those involved in defence. Cheng, Sullivan and Harris (1973) reported that the marine gastropod *Nassarius obsoletus* was castrated by chemicals secreted by the sporocysts of *Zoogonius rubellus* and that were specific for germinal epithelium and gametes. Studies on the freshwater snail *Lymnaea stagnalis* infected with *Trichobilharzia ocellata* have shown that substances secreted by the trematode induce changes in host gene expression to directly inhibit mitotic division in the male copulatory organ and also stimulate development of the female endocrine dorsal bodies (De Jong-Brink, Bergamin-Sassen and Solis-Soto, 2001). Likewise Rice *et al*. (2006) showed that infection of the mollusc *Haliotis asinina* by the trematode *Allopodocotyle* sp. results in parasitic castration and is accompanied by differential expression of a number of regulatory genes. Manger, Christensen and Yoshino (1996) found that *Schistosoma mansoni* appropriated, for its own use, the hosts neurotransmitters serotonin and dopamine leading to changes in the host *Biomphalaria glabra* endocrine system.

Perkinsus marinus, P. olseni and *Haplosporidium nelsoni* have all been shown to affect the growth and condition of their host molluscs which led Flye-Sainte-Marie *et al*. (2007) to demonstrate that brown ring disease of clams *(Ruditapes philippinarum*) caused by the bacteria *Vibrio tapetis* affected the energy budget of the clams and resulted in a reduction of the clearance and respiration rate, possibly due to the energy requirements associated with the immune response and tissue repair. How many published studies of the physiology of molluscs have been compromised because the disease status of the animals was not considered a factor?

Parasite-environment interactions

a) Impact of pathogen on the environment of the host

It is known that parasite mortality events can alter the host density leading to major changes in the ecology of an area. *Marteilia* sp. infections lead to the commercial extinction of *Ostrea edulis* from the Gulf of Thessalonaiki (Virvilis and Angelides, 2006). Also, Miura *et al.* (2006) showed that the mud snail *Batillaria cumingi*, when infected by the trematode *Cercaria batillariae*, develop a different morphological form, move to the lower intertidal zone and consume different resources from uninfected snails. The parasites are, thus, indirectly altering the food web of many marsh species.

b) Impact of environment on pathogen

Studies on the effect of the environment (including pollution) on the susceptibility of the host to the pathogen have been done, particularly for MSX (*Haplosporidium nelsoni*) in oysters in the United States of America (USA), but studies on other pathogens and their hosts are more limited. Hégaret *et al*. (2007) showed that infection of clams (*Rudetapes philippinarum*) with *Perkinsus olseni* had no measurable effect on haemocyte parameters measured, but when the clams were exposed to toxic algal blooms there was a measurable change in haemocyte parameters monitored, and thus immunomodulation, in heavily infected clams exposed to toxic algae when compared to heavily infected clams that were not exposed. While we know a lot about the effects of the environment on the mollusc immune system, we know very little

about the effect of the environment on the biochemistry of the pathogen when it is in the host. The optimal conditions for the host may not be those of the pathogen, such that changes in the environment (salinity, temperature) may place the pathogen at a disadvantage.

Research has tended not to disengage the effects on the external infectious stages from the impact of the environment on established infections. For example, the probability of a severe kill due to winter mortality (*B. roughleyi*) is higher after dry autumns and early winters. Growers minimise losses by relaying oysters to low salinity upstream locations during periods of potential infection. Whether these actions are mitigating the effects of subclinical infections, perhaps by reducing parasite replication in the host, or are avoiding new infections by breaking the life cycle has apparently not been studied.

Environmental pollution affects both host communities and parasite populations. Again, there has been much recent work done on the effect of pollutants on the host, and recognition in the literature that parasite communities are a potential indicator of environmental disturbance but there has been little study on the effect of pollutants on the parasite itself.

Changes in parasite abundance and prevalence have also been used as an environmental monitor. Ectoparasites tend to increase and endoparasites decrease in prevalence and abundance in fish after chronic exposure to xenobiotics and aromatic hydrocarbons (MacKenzie, 1999; Kahn, 2004), and the impact on invertebrate hosts is likely to be similar, leading to establishment of monitoring programs such as the USA's "mussel watch" programme (Kim *et al.,* 2008). Contaminants may favour the propagation of parasites by excluding predators, reducing host resistance, improving living conditions of host, or may interfere with parasite biochemistry thus reducing parasite burden or pathogenicity.

Environment-host interactions

a) Non-pathogenic diseases directly induced by environmental changes and pollution. Examples include tumours, toxins, endocrine disruptors and other non-infectious diseases. Describing tumours in molluscs has a long history starting with Ryder (1883), see also subsequent reviews by Pauley (1969), Farley and Sparks (1970), Elston, Moore and Brooks (1992), and Sparks (2005). More recently there has been a growing body of literature on the tissue effects of pollutants, particularly heavy metals, on molluscs following on from the work on tributyl tin antifouling and the imposex[5] that it causes (Smith, 1981; Tallmon and Hoferkamp, 2009). Work has also focussed on oil pollution and endocrine disruption associated with veterinary and medical drug residues (Marigomez *et al.,* 2006; Matthiessen, 2008; Morley, 2008). There has been a parallel growth in papers researching the use of mollusc diseases as a bioindicator of environmental pollution (see review by Au, 2004).

[5] Imposex is a descriptive term applied to some seasnails, marine gastropod molluscs which, under the toxic effects of pollutants, develop sex organs that are in contrast to their actual sex. It is a pathological condition where male sex characteristics, such as the development of male sex organs, (for example the penis and the vas deferens) form in female gastropods (http://en.wikipedia.org/wiki/Imposex)

b) Changes to host susceptibility driven by the environment, leading to disease
Environmental changes do not have to damage tissues and interfere with biochemical processes to affect oyster immune status. More subtle environmental changes are often classified as 'stress' and include, but are not limited to: mechanical disturbance (Lacoste *et al.,* 2002; Ballarin, Pampanin and Marin, 2003); salinity changes (Fisher, Auffret and Ballouet, 1987; Butt, Shaddick and Raftos, 2006); temperature (Soudant *et al*., 2004; Cheng *et al*., 1975; Cheng *et al.,* 2004; Zhang *et al*., 2006); chemical pollution (Pipe and Coles, 1995; Oliver *et al*., 2001; Cheng, Hsiao and Chen, 2004; Cheng, Juang and Chen, 2004) and diet including starvation (Butt *et al*., 2007). For example, Hong Chen *et al*. (2005) found that both phagocytosis and phenoloxidase activity in *Haliotis discus hannai* were affected by lack of dietary pyridoxine (vitamin B6). It is also certain that the enzyme systems of molluscs will be adapted to optimally perform at the normal temperature range. Changes in temperature may change host biochemistry thus favouring pathogens.

The 1986 *Bonamia* epizootic in Foveaux Strait, New Zealand, began in areas that had been fished intensively for years and where benthic habitat was highly modified (Cranfield, Michael and Doonan, 1999). Similar escalating disease mortality in oysters in Chesapeake Bay, USA, has been attributed to modification of oyster habitat by fishing (Rothschild *et al.,* 1994), and the environmental stress caused by this modification has been directly implicated in increasing acetosporan disease levels of oysters in experiments (Lenihan *et al*., 1999).

CONCLUSIONS

It is not only our knowledge about known mollusc diseases that has grown. New diseases continue to be regularly reported as aquaculture becomes more intensive; as the Asia and the Pacific regional skills base develops and more diseases are reported; as international reporting also becomes more accurate; and as transfer of disease between jurisdictions becomes more rapid as products are sent live - both as broodstock and as products for human consumption (such as the spread of *Bonamia* spp. infected oysters throughout Europe).

There is a need for an increase in the numbers of trained diagnosticians as well as those investigating the ecology of diseased molluscs at a local level. It has been recognised by agencies such as the Network of Aquaculture Centres in Asia/Pacific and the Food and Agriculture Organization of the United Nations that the skills base needs to be developed at three levels: Level I (farm/production site observations, record-keeping and health management) is strongly emphasized throughout the *Asian Diagnostic Guide (*Bondad-Reantaso *et al*., 2000*)* as this forms the basis for triggering the other diagnostic levels (II and III). Level II includes specialisations such as parasitology, histopathology and bacteriology that, generally speaking, cannot be conducted at the farm or culture site. Level III comprises advanced diagnostic specialisation that requires significant capital and training investment, such as TEM. Immunology and biomolecular techniques are included in Level III, although field kits are now being developed for farm or pond-side use (Level I) as well as use in microbiology or histology laboratories (Level II). These efforts are good indication that technology transfer is now enhancing diagnostics and, with solid quality control and field

validation, it is certain that more Level III technology will become field accessible in the near future (Walker and Subasinghe, 2000).

It is inevitable that, as the initial work on mollusc diseases developed around shellfish growing areas in Europe and America, the next generation of molluscan disease experts will be based in the Asia Pacific region.

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Lysozymes in molluscs

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ABSTRACT

Invertebrates lack antibody-mediated humoral immune systems; however, they are believed to possess efficient host defense mechanisms involving humoral defense molecules that are similar in function to antibodies. Lysozymes are a group of enzymes that cleave the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine (two amino sugars) in the peptidoglycans that form bacterial cell walls. In bivalves, lysozymes are especially important antibacterial molecules because of their bactericidal ability. Recently, the presence of multiple lysozymes has been found in several species of bivalve molluscs such as the blue mussel, *Mytilus edulis*, and the eastern oyster, *Crassostrea virginica*. Therefore, to determine the molecular and biochemical properties of bivalve lysozymes, we have identified the cDNA sequences of three different lysozymes (CGL-1, -2, and -3) from the Pacific oyster, *C. gigas*, and have produced recombinant lysozymes (rCGL) using the methylotrophic yeast *Pichia pastoris*. The lysozyme CGL-1 mRNA was expressed in all tissues except for those of the adductor muscle. In contrast, CGL-2 gene was only expressed in digestive diverticula. Interestingly, in digestive diverticula, CGL-1 gene expression was detected in the same digestive cells as that of CGL-2. It is therefore possible that CGL-1 and CGL-2 play complementary roles in digestive organs. *In situ* hybridization revealed that CGL-3 mRNA was highly expressed in the mantle and haemocytes. These results suggest that CGL-1 and -2 serve as digestive enzymes for enteric and engulfed bacteria in digestive organs and that CGL-3 is involved in biodefense against invading microbes. Based on the results from experiments using recombinant lysozymes, we found significant differences among the characteristics of the three lysozymes, suggesting that these lysozymes have different functions in *C. gigas*.

Key words: biodefense mechanism, lysozyme, oyster, *Crassostrea gigas*, bivalve molluscs

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INTRODUCTION

Bivalve molluscs and microorganisms coexist in the biosphere in numerous ways. Thus, bivalves have evolved sensitive mechanisms for recognizing pathogens and an array of strategies to defend themselves against attacks by microorganisms such as bacteria, fungi, and parasites. However, bivalve molluscs, as well as other invertebrates, lack an antibody-mediated adaptive immune system. They have an innate immune system that comprises haemocytes and non-specific humoral defense molecules. Therefore, to combat infection, bivalves rely on multiple defense reactions. The point of biodefense mechanisms is to recognize and eliminate various types of pathogens. Some defense molecules are able to respond to typical microbial pathogen-associated molecular patterns (PAMPs), and these molecules are used to distinguish pathogens from host cells. In insects, for example, peptidoglycan recognition proteins (PGRPs) play a crucial role in the recognition of bacterial pathogens and in the induction of various immune reactions. However, in bivalve molluscs, although the genes of PGRP have been cloned and sequenced from the scallops *Argopecten irradians* and *Chlamys farreri* (Ni *et al.*, 2007; Su *et al.*, 2007) and from the Pacific oyster, *Crassostrea gigas* (Itoh and Takahashi, 2008; 2009), their functions are still unclear. Although humoral responses to bacterial pathogens remain unknown in bivalves, it is considered that lectins might be the main recognition molecules as they function as a binding receptor for pathogens and as an opsonin for phagocytosis by haemocytes (summarized by Muroga and Takahashi, 2007). Lysozymes are a cleaving enzyme that break the glycosidic bonds in bacterial peptidoglycans, and these enzymes have been found in many species of bivalves and are considered to be one of the most important types of defense molecule.

BRIEF REVIEW ON BIVALVE MOLLUSC LYSOZYME STUDIES

Lysozymes (EC 3.2.1.17) occur in a wide variety of cells, tissues, and secretions from bacteriophages to mammals. They are a family of glucoside hydrolases that cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycans forming bacterial cell walls. Thus, lysozymes are bacteriolytic enzymes and play a major biological role in biodefense, as these enzymes can act as antibacterial and immune-modulating agents. In addition, lysozymes function as important digestive enzymes in some animals. Since Mcdade and Tripp (1967a, b) reported the detection of lysozyme activity in the hemolymph and skin mucus from the American oyster, *C. virginica*, lysozyme and lysozyme-like activity have been found in various bivalve molluscs (summarized by Muroga and Takahashi, 2007).

Three families of lysozymes have been identified in animals, they are the chicken type (c-type), goose type (g-type), and a new type of lysozyme; i.e., the invertebrate type (i-type). The existence of the i-type lysozyme was first proposed as a new type of lysozyme based on analysis of the quantitative amino acid composition and the N-terminal sequence of a lysozyme from the common starfish, *Asterias rubens* (Jollès and Jollès, 1975). In the late 1990s, a lysozyme from the clam *Tapes japonica* (*Ruditapes philippinarum*) was isolated, and its complete amino acid sequence at the protein level was determined

(Ito *et al*., 1999). This was the first bivalve mollusc lysozyme to be identified as an i-type lysozyme. Subsequently, cDNA coding lysozymes have been cloned and sequenced from the Icelandic scallop, *Chlamys islandica* (Nilsen *et al*., 1999; Nilsen and Myrnes, 2001) *T. japonica* (Takeshita *et al*., 2004), *C. gigas* (Matsumoto, Nakamura and Takahashi, 2006; Itoh and Takahashi, 2007), *C. virginica* (Itoh *et al*., 2007; Xue *et al*., 2007), and the Mediterranean mussel, *Mytilus galloprovincialis* (Li *et al*., 2008). Alignment and phylogenic analyses using six bivalve lysozymes have demonstrated that i-type lysozymes form a monophyletic family (Bachali *et al*., 2002). The gene of a lysozyme from *C. islandica* (designated chlamysin) was shown to have four exons of 38 to 252 bp separated by large introns (Nilsen and Myrnes, 2001). The overall gene organization of chlamysin resembled that of the c-type lysozyme genes found in invertebrates, but it differed from the invertebrate c-type lysozyme genes by having a three-exon structure. Furthermore, based on crystal structure and mutation analyses of *T. japonica* lysozyme (TJL), Goto *et al*. (2007) demonstrated that TJL formed a dimer by electrostatic interactions between catalytic residues (Glu-18 and Asp-30), and that the TJL dimer (inactive form) remained intact under low salt concentrations but that it dissociated into TJL monomers (active form) under high salt conditions. Moreover, with increasing salt concentrations, the chitinase activity of TJL markedly increased. In general, the lytic activity of bivalve lysozymes has been shown to be sensitive to the ionic strength and salt concentration of solutions (Muroga and Takahashi, 2007).

By using enzymatic analyses, the functions of bivalve lysozymes were estimated as digestion (McHenery and Birkbeck, 1979; McHenery, Birkbeck and Allen 1979; Jollès, Fiala-Médioni and Jollès, 1996) and host defense (Jollès and Jollès, 1984). Bacteria are the chief source (nitrogen and phosphorous) of food in bivalve molluscs as well as in other invertebrates. Bivalve molluscs are also classically known to feed through heterotrophic processes on marine bacteria and/or other planktonic organic materials filtered from seawater. In addition, Jollès, Fiala-Médioni and Jollès (1996) suggested a digestive role for the lysozymes in deep-seawater bivalve molluscs, which rely on symbiotic bacteria in their gills for nutrition. In fact, digestive lysozymes from many bivalve species were isolated or purified from the digestive glands and crystalline styles, e.g., *M. edulis* (McHenery and Birkbeck, 1979; McHenery, Birkbeck and Allen, 1979; Olsen *et al*., 2003); *T. japonica* (Ito *et al*., 1999); *C. virginica* (Xue *et al*., 2007); *C. islandica* (Nilsen *et al*., 1999); and the brackish water clam *Corbicula japonica* (Miyauchi, Matsumiya and Mochizuki, 2000). The crystalline style is a rod-shaped organ in the bivalve stomach in which various digestive enzymes are stored in high concentrations. In *T. japonica*, TJL may be rendered inactive by dimer formation in the crystalline style under highly concentrated conditions (Goto *et al*., 2007). When bivalves feed, they ingest bacteria present in the seawater, which has a high salt concentration. With increasing salt concentration in the stomach, the inactive TJL dimer is converted to the active TJL monomer (Goto *et al*., 2007). Recently, the presence of multiple lysozymes with different biochemical properties has been demonstrated in *M. edulis* (Olsen *et al*., 2003) and *C. virginica* (Xue *et al*., 2004; Xue *et al*., 2007). A *C. virginica* lysozyme purified from plasma (CVL-1) was found to be unique in its N-terminal amino acid sequence and showed optimal activity at high ionic strength (Xue *et al*., 2004). CVL-1

possesses strong antimicrobial activity, which suggested that its main role is in host defense (Itoh *et al*., 2007). Furthermore, a different lysozyme, designated CVL-2, was purified from the crystalline styles and digestive gland in *C. virginica* (Xue *et al*., 2007). CVL-2 showed high amino acid sequence similarity to other bivalve lysozymes, but its biochemical and molecular properties, distribution in the oyster body and site of gene expression suggested that its role was in digestion (Xue *et al*., 2007). These facts indicate that each species of bivalve mollusc possesses multiple lysozymes in various tissues such as the hemocytes and plasma or the digestive gland and crystalline style and may use different lysozymes for different functions (host defense or digestion, for example). These findings also indicate that the application of bacteria lysis tests using *Micrococcus luteus* (*lysodeikticus*) in classical studies is of limited use for characterizing unpurified lysozyme because a mixture of multiple lysozymes might be measured and characterized as lysozyme activity. However, it is never made particularly clear what that distinction between digestive and immune lysozymes. Thus, to better understand the physiological roles of lysozymes, it is necessary to identify each lysozyme molecule and determine its molecular and biochemical properties.

RECENT ADVANCES IN THE FIELD OF OYSTER LYSOZYMES AND THEIR ROLE IN BIODEFENSE MECHANISMS

The Pacific oyster, *C. gigas*, is the most important commercial species in the world and is a physiologically well-studied bivalve species. The lysozyme activity of *C. gigas* was first recognized by Mori *et al.* (1980) as bactericidin. Takahashi, Mori and Nomura (1986) concluded that lysozyme is a chief component of bactericidin, and Matsumoto, Nakamura and Takahashi (2006) identified a cDNA sequence of lysozyme (CGL-1) from *C. gigas*. However, it remained unknown whether *C. gigas* possesses multiple lysozymes, and it was still unclear whether the lysozymes in *C. gigas* were able to function as a digestive enzyme and/or as a host-defense molecule. Thus, to clarify the function of lysozymes in host defense mechanisms in *C. gigas*, we have been investigating molecular and biochemical characteristics of lysozymes since 2007. We have attempted to clone and identify each lysozyme cDNA; to demonstrate sites of lysozyme gene expression; to produce recombinant lysozymes; to characterize the properties of recombinant lysozymes; to purify each lysozyme molecule; and to determine the tissue distributions of each lysozyme molecule.

We obtained and identified a second lysozyme cDNA from the digestive gland of *C. gigas* (Itoh and Takahashi, 2007). A 536-bp of cDNA encoding a novel lysozyme was identified and designated as CGL-2. The encoded lysozyme is comprised of 142 amino acid residues including 20 residues of a signal peptide. The amino acid sequence of CGL-2 was different from that of CGL-1. CGL-1 was expressed in multiple tissues of *C. gigas*, especially the mantle, hemocytes, and digestive gland (Matsumoto, Nakamura and Takahashi, 2006). The CGL-2 has a lower isoelectric point and fewer peptide recognition sites; *in situ* hybridization revealed CGL-2 mRNA transcription in the digestive cells of the digestive tubule, suggesting that CGL-2 has evolved to adapt to digestive environments. Moreover, CGL-1 gene expression was also detected in the same digestive cells as CGL-2 (Itoh and Takahashi, 2007). It is predicted that CGL-1 and CGL-2 play complementary roles

in the digestive organs in *C. gigas*. To investigate the biochemical characteristics of each lysozyme, we produced recombinant CGL-1 and CGL-2 (rCGL-1 and rCGL-2) using the methylotrophic yeast *Pichia pastoris*. rCGL-1 showed relatively high activity within a broad range of pH and ionic strengths (Fig. 1). The maximum activity of rCGL-1 was detected in salt concentrations in excess of 150 mM. In contrast, rCGL-2 expressed more than 90% of its maximum activity in the pH range of 5.4 to 6.4 when the salt concentration was very low (Fig. 1). rCGL-2 activity was almost completely inhibited at higher ionic strengths.

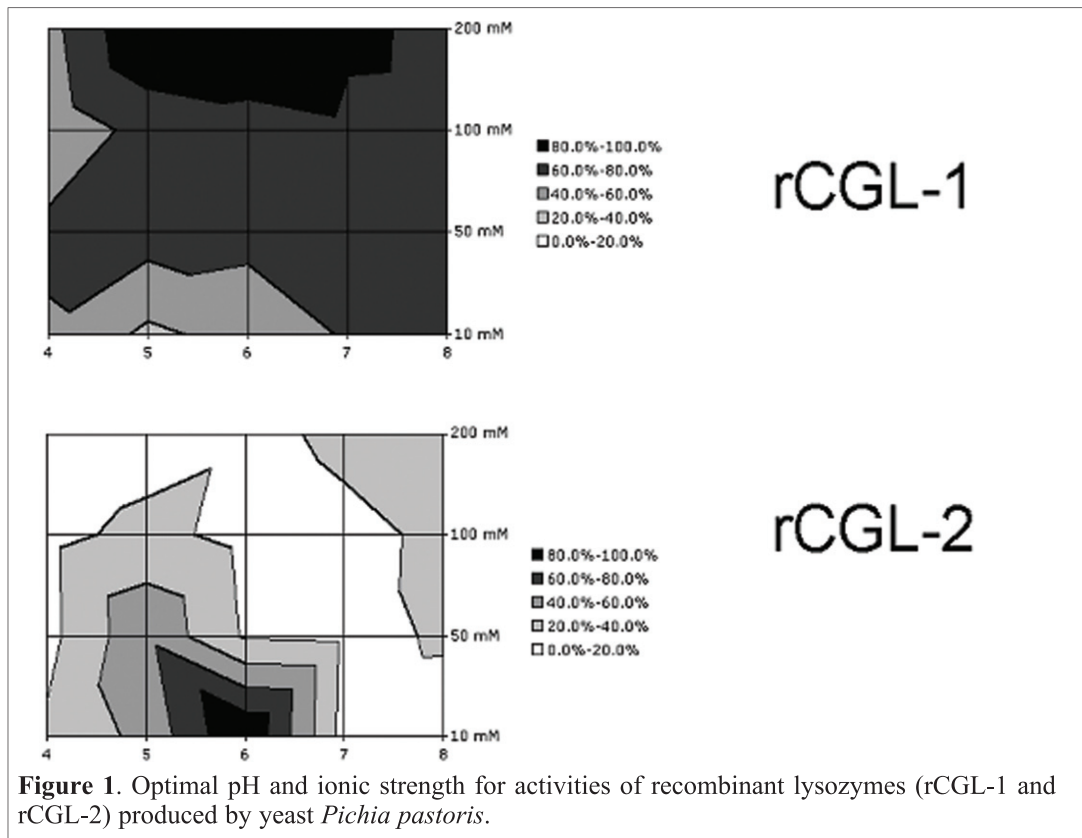

Figure 1. Optimal pH and ionic strength for activities of recombinant lysozymes (rCGL-1 and rCGL-2) produced by yeast *Pichia pastoris*.

We also cloned and identified a third lysozyme cDNA from the mantle tissues in *C. gigas* (Itoh and Takahashi, unpublished). This lysozyme was designated CGL-3. A protein-protein BLAST search in GenBank revealed that the amino acid sequence deduced from the CGL-3 cDNA is homologous to bivalve i-type lysozymes (Fig. 2). CGL-3 has a signal peptide sequence comprising 20 residues, and mature CGL-3 consists of 122 amino acid residues. Furthermore, multiple alignments with bivalve i-type lysozymes showed that the important residues responsible for lysozyme activity, Glu34, Asp45 and Trp61, were present in CGL-3. Thus, it is considered that all three lysozymes, CGL-1, CGL-2, and CGL-3, have true lysozyme activity in the body of *C. gigas*. We also measured the antibacterial activities of the three recombinant lysozymes. Results are expressed as the minimum concentration (MIC) of lysozymes that significantly inhibited bacterial growth.

As shown in Table 2, antibacterial activity differed greatly among the three lysozymes. rCGL-3 revealed the strongest activity, as it significantly inhibited the growth of *Escherichia coli* at a concentration of 1.6 µg/ml. rCGL-2 was significantly less effective than both rCGL-1 and rCGL-3. A comparison of the molecular and biochemical characteristics of CGL-1, CGL-2, and CGL-3 is shown in Table 1.

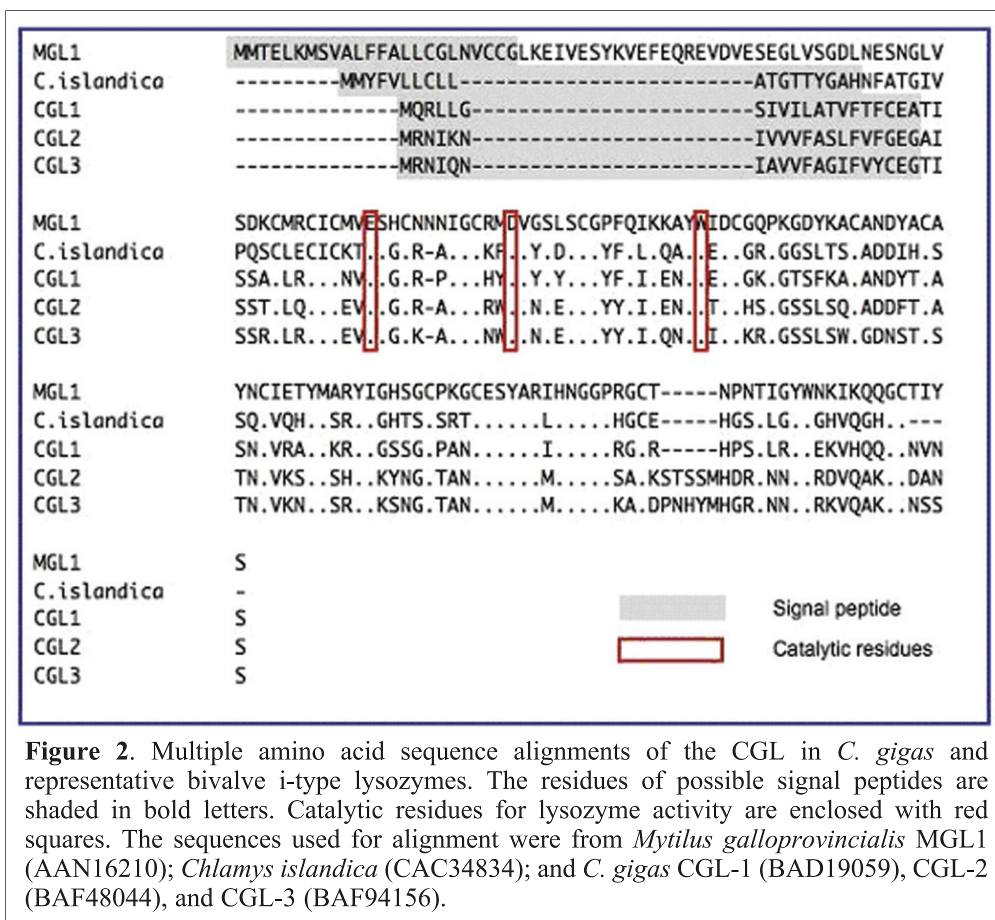

Figure 2. Multiple amino acid sequence alignments of the CGL in *C. gigas* and representative bivalve i-type lysozymes. The residues of possible signal peptides are shaded in bold letters. Catalytic residues for lysozyme activity are enclosed with red squares. The sequences used for alignment were from *Mytilus galloprovincialis* MGL1 (AAN16210); *Chlamys islandica* (CAC34834); and *C. gigas* CGL-1 (BAD19059), CGL-2 (BAF48044), and CGL-3 (BAF94156).

Significant differences were found in the characteristics of the three lysozymes, suggesting that they have different functions in *C. gigas*. Firstly, CGL-2 was mainly expressed in the digestive gland. Secondly, the lower isoelectric point of CGL-2, compared to those of CGL-1 and CGL-3, suggests that CGL-2 functions in an acidic environment. Thirdly, the number of arginine residues and protease cutting sites in CGL-2 was lower than in the other two lysozymes, suggesting that CGL-2 has an increased resistance to proteolytic digestive enzymes and has adapted to digestive conditions. Thus, we concluded that the main role of CGL-2 is related to digestion. In contrast, CGL-1 showed high activity in relatively high salt concentrations, suggesting that CGL-1 is better suited to function in the mantle tissues and the hemolymph, where pH and salt concentrations are usually high. Therefore, it is suggested that CGL-1 functions as both a host defense molecule and a digestive enzyme. CGL-3 was

Table 1
Comparison of CGL-1 molecule characteristics with those of CGL-2 and CGL-3

	CGL-1	CGL-2	CGL-3
Molecular weight	13079.5	13494.7	13698.2
Predicted pl	8.03	6.03	8.27
Total AA residues	117	122	122
No. argenine residues	8	5	7
Protease cutting sites			
Trypsin	1	10	14
Thermolysin	22	18	18
Tissue expression	Multiple	Digestive gland	Mantle
Optimal salinity	>150mM	<20 mM	ND

Table 2
Antibacterial activity of recombinant CGL

Antibacterial activity (MIC, µg/ml)	rCGL-1	rCGL-2	rCGL-3
Escherichia coli	12.5	50	1.6
Vibrio tubiashii	50	100	100
Marinococcus halophilus	0.8	2.5	0.8

found to be mainly synthesized in the mantle and hemocytes. In addition, CGL-3 showed the highest antibacterial activity among the three lysozymes. Thus, we propose that the main role of CGL-3 is related to host defense. However, we were unable to elucidate the function of each lysozyme in *C. gigas* because it is possible that the lysozymes can perform more than one function simultaneously.

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Investigating a mortality in hatchery cultured tropical abalone, *Haliotis asinina* Linnaeus, 1758 in Malaysia

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ABSTRACT

Abnormal mortality rates ranging from 4-5 individuals/day were reported for abalone cultured in fibreglass tanks at the hatchery facility of the Fisheries Research Institute, Penang, Malaysia. Previously, mortality was only in the range of 1-3 abalone/month. Histological and bacteriological examination of the samples collected over a three-month period showed systemic bacterial infection by Gram-negative bacterial rods with a presumptive identification to the genera *Vibrio* spp. and *Pasteurella* spp. The main histological feature observed was severe enteritis. Further investigation confirmed that the disease was transmitted from the seaweed (*Gracilaria changii*) used as food for the abalone. The *Gracilaria changii* stock was procured from abandoned shrimp ponds located on the north western coasts of the peninsula. This case study highlights the importance of good farming and management practices and as well as appropriate abalone husbandry procedures.

Key words: tropical abalone, *Haliotis asinina*, vibriosis, good management practices, *Gracilaria changii.*

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INTRODUCTION

Abalone are among the top five most expensive food items on the Chinese cuisine market. They are usually sold fresh, frozen, canned or dried and are consumed raw (sashimi) or cooked. Although abalone are slow-growing molluscs, they are a new candidate for aquaculture in many countries as wild fisheries have progressively been declining. These factors have prompted the Fisheries Research Institute (FRI) in Malaysia to embark on an abalone breeding program which was first initiated in 2000.

Haliotis asinina L. is a tropical abalone species, commonly known as the donkey's or ass`s ear abalone. It is native to Australasia, Thailand, the Philippines, Malaysia, Vietnam and New Zealand. Despite its moderate size (8-10 cm shell length) and dubbed the 'cocktail abalone', it is a species with great potential for aquaculture in Southeast Asia. Hatchery propagation and farming of this species has been proven to be successful in Thailand, the Philippines, Australia, New Zealand and recently in Malaysia.

Adult *H. asinina* broodstock were procured from the northern waters of East Malaysia, off Sabah (from Kota Belud, Pulau Matanani and off Sempoerna Island). They were air freighted to Penang, and subsequently transported by land to the mollusc hatchery at FRI. Upon arrival the abalone were conditioned in 2-tonne (2000 l) fibreglass tanks. The adults spawn after about 2-3 months of conditioning. These tropical abalones need not be induced to spawn as they spawn naturally coinciding with the lunar cycles (new and full moon period). The larvae settle within 2-4 days and they feed on pre-cultured diatoms collected on acrylic culture plates. The abalone spat usually take about 18 months to attain marketable size of 6-7 cm. During the culture period, increased mortality rates were noted for both the adult and juvenile abalones. This paper reports the findings of the study conducted to investigate the cause of mortality of *H. asinina* in the rearing facility.

MATERIALS AND METHODS

Rearing conditions – Abalone broodstock were held in 2000 l tanks with flow-through sea water (4 l/min) at a stocking density of 200 adult abalone (6-8cm/tank). Juveniles were stocked at 1,500 pieces per 2000 l tank. The hatchery facility consisted of six 2000 l tanks: three tanks for adult abalone and three tanks for juveniles. The adults were fed seaweed, *Gracilaria changii* while the juveniles were fed seaweed and mixed diatoms. The seaweed stock was held in a separate tank and used on demand to feed the abalone. Similar rearing conditions were provided for the adults and the juveniles: tanks were supplied with flow-through seawater and provided aeration. Water change was carried out at 50% and 100% on alternate days. Every fortnight (i.e. after 14 days), the abalone juveniles were transferred manually, using a hard plastic strip to loosen their grip, into a new tank with fresh diatoms. Waste was siphoned out routinely and uneaten seaweed was collected and washed before returning it to the culture tanks.

Case history – An unusually high mortality rate was reported in January 2005. The facility management noted 4-5 abalone dying daily as compared to only 1-3 dying monthly, previously. Healthy and sick individuals were collected on the 17th and 23rd February and 21st March, 2005. Sampled abalone were subjected to clinical observation and further bacteriological isolation and histological examination.

Investigation - Fresh smears from the gills and tegument lesions were taken to assess parasitic infections. Necropsies were conducted under a dissecting microscope (4 – 40X magnification).

Primary isolations of bacteria from the lesions on abalone and the seaweed (*Gracilaria changii*) were carried out on Tryptone Soya Agar, TSA (OXOID) with additional 1.5% salt for total heterotrophic flora and Thiosulfate Citrate Bile Sucrose agar, (TCBS) (OXOID) for *Vibrio* spp. at ambient temperature (30°C). The plates were incubated at 30°C for 24 to 48 hrs. The bacteria isolated were identified using the API 20E kit (BioMé reiux).

Normal and abnormal specimens were dissected and lesions from the foot margin, ulcer-type lesions from the tegument and samples of 1 cm thickness of the cross-sectioned internal organs consisting of gonad, gills, intestine and stomach, were fixed in 10% buffered formalin in filtered seawater for 24-48 hrs, followed by storage in 70% alcohol. Samples were subsequently processed for histology according to standard methods (Bondad-Reantaso *et al.*, 2001), stained with either hematoxylin-eosin (H&E), Gram's stain or methylene blue and mounted for light microscopy (Zeiss Axioplan). .

RESULTS

The mortality rate was 4-5 abalone/day over a six-month period, resulting in high cumulative mortality. Affected individuals were generally weak with the following clinical signs:

i) The mantle was attached loosely to the shell with development of a white pseudo-membrane in some instances (Fig. 1a).

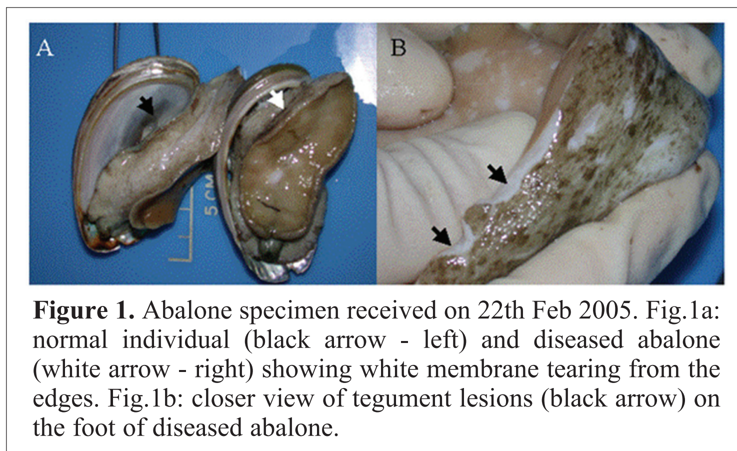

Figure 1. Abalone specimen received on 22th Feb 2005. Fig.1a: normal individual (black arrow - left) and diseased abalone (white arrow - right) showing white membrane tearing from the edges. Fig.1b: closer view of tegument lesions (black arrow) on the foot of diseased abalone.

ii) Some specimens showed ulcer-like lesions of the tegument. In the most severe cases, individuals displayed white patches at the foot margins (Fig. 1b).

iii) No parasites were found from fresh smears and squashes. Gram-negative bacteria, presumptively identified as *Pasteurella* spp. and *Vibrio* spp. were isolated from the foot lesions (Fig. 1b). and characterised by biochemical tests (Table 1).

Table 1
Biochemical tests on the Gram-negative bacteria isolated from the foot lesions of abalone

Gram negative bacteria		
Test	*Pasteurella* species	*Vibrio* species
TSA	Yes	Yes
Colony colour on TCBS	Green	Yellowish
Gram stain	Negative	Negative
Shape	Rod	Rod
Oxidate/Fermentative	Positive/Positive	Positive/Positive
Motility	Negative	Positive
Oxidase	Positive	Positive
McConkey	Negative	Negative
0/129	Sensitive	Resistant
API20E	*Pasteurella pneumo/haemol*	*Vibrio alginolyticus*

iv) Gram-negative bacteria identified as *Vibrio* spp. (*Vibrio vulnificus* and *Vibrio alginolyticus*) were isolated from the seaweed.

Histology revealed systemic bacterial infection with intense haemocytic infiltrations and abscess-like lesions in the foot (Fig. 2). Some haemolymph sinuses were abnormally dilated with haemocytes adhering to the vessel wall. This was interpreted as an early sign of a

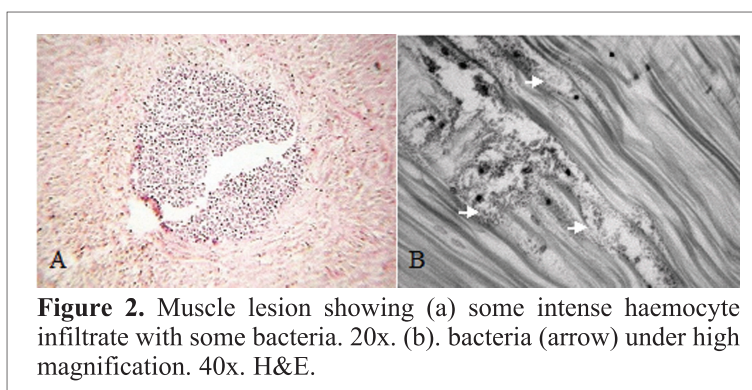

Figure 2. Muscle lesion showing (a) some intense haemocyte infiltrate with some bacteria. 20x. (b). bacteria (arrow) under high magnification. 40x. H&E.

systemic inflammatory response (Fig. 3). Inflammation was also noted in the nervous tissue. Some specimens had severe hemorrhagic enteritis associated with septicaemia.

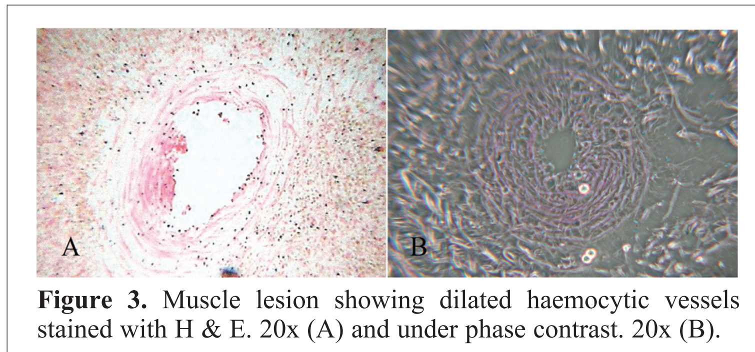

Figure 3. Muscle lesion showing dilated haemocytic vessels stained with H & E. 20x (A) and under phase contrast. 20x (B).

DISCUSSION

The high mortality rate recorded in the rearing facility led to a comprehensive investigation of the possible origin and remediation options. The investigation concluded that the outbreak was caused by bacterial infections of *Vibrio* and *Pasteurella*. Histopathological examination revealed abscesses, enteritis and severe haemocyte infiltration in response to systemic bacterial infection.

Bacterial infections of abalone are well documented. Vibriosis has been reported to be caused by *Vibrio harveyi, V. splendidus* I, and *V. alginolyticus* (Dixon *et al*., 1991; Elston and Lockwood, 1983; Handlinger *et al*., 2002; Reuter and McOrist, 1999; Lee *et al*., 2001; Nishimori *et al*., 1998). Among these, *V. harveyi* and *V. carchariae* (a junior synonym of *V. harveyi*, see Pedersen *et al*., 1998) are more prevalent. Nishimori *et al*. (1998) reported mass mortalities in Japanese abalone *Haliotis diversicolor supertexta* Lischke, 1870 due to *V. harveyi.* Bacterial infection such as *V. harveyi* in *H. tuberculata* L. has been reported in both wild and cultured abalone in Brittany and Normandy, France (Nicolas *et al*., 2002). In Tasmania, Australia, *V. harveyi* and *V. splendidus* I were found in abalone showing septicaemia. In addition, *Flavobacterium*-like bacteria have been identified during disease outbreaks in cultured *H. rubra* Leach, 1814, *H. laevigata* Donovan, 1808 and their hybrids (Matsunaga, 1967). *Clostridium lituseberense* has also been reported to occur in abalone (Bower, 2003 and 2004). However, infection by *Pasteurella* sp. in abalone has not previously been reported. In this case, *H. asinina* L. with necrosis and white lesions on the foot had shown infection caused by two bacteria types, presumptively identified as *Pasteurella* sp and *Vibrio* spp. In this study, we were able to identify the bacteria up to the genus level, however, confirmation of the bacterial species needs to be pursued in future.

The mortality could be associated with poor management practices in the hatchery, such as direct use of seaweed from the prawn farms (introduction of anthropogenic pollutants such as bacteria, parasites and mud) and returning uneaten seaweed to the tanks which

further fouled up the water due to presence of waste matter. The manual removal of the abalone could also have inflicted lesions on the body. No specific treatment was given at the time of the mortalities. Mitigation measures undertaken were frequent exchange of water in the abalone and seaweed culture tanks and quarantine of the seaweed prior to feeding, which showed a notable decrease in the number of mortalities.

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A Reo-like virus associated with high mortality rates in cultured mud crab, *Scylla serrata*, in East China

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ABSTRACT

The discovery of a reo-like virus in cultures of the mud crab *Scylla serrata* (Forsskål, 1755) is reported here. Since May 2004, in Zhejiang Province, Peoples Republic of China, reo-like virus in mud crabs has reached serious epidemic levels, causing mortality rates exceeding 90%. Beginning in May 2004, moribund crabs from different districts and areas were investigated. By using electron microscopy, two different organisms could be detected: reo-like viruses and mollicute-like organisms. The complete viral particle is 60 nm in diameter, icosahedral and non-enveloped. The viruses infect the cytoplasm of connective tissue cells of the gill, stomach, heart and intestine of mud crabs.

Key words: *Scylla serrata,* virus, mollicute, electron microscopy

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INTRODUCTION

Mud crab, *Scylla serrata* (Forsskål, 1755), traditionally called 'green crab', is an economically important marine aquaculture species cultured in the People's Republic of China. In the past few years the aquaculture industry based on *S. serrata* has experienced rapid growth, but in the meanwhile the industry is facing increasing economic losses caused by outbreaks of various diseases. Disease has become one of the main factors inhibiting the development of *S. serrata* aquaculture in Zhejiang Province, China with survival rates less than 10%.

Since summer 2004, an epidemic disease has broken out in Sanmen County and its adjacent areas with symptoms differing from a previous disease outbreak called 'yellow water disease'. These symptoms included debility, weak grip strength of pincers and feet, hydroabdomen, white colour in carapace, drying of gill filaments, and water-like blood after breaking the feet (blood coagulation capacity is weak). Moreover, the disease is widely spread, and is fatal to commercial size crabs, fingerings (40-60 individuals /kg) and breeding crabs. Antibiotic drugs had no effect in relieving the symptoms. The disease is temporarily termed 'clearwater disease' (CD) due to its major symptom. In this study, the ultrastructure of infected crab tissues collected in Sanmen County in 2004 and 2005 were investigated. Two different pathogens in moribund crabs were detected: reo-like viruses and mollicute-like organisms.

MATERIALS AND METHODS

Mud crab
Five moribund mud crabs were obtained from a mud crab farm in Sanmen County, Zhejiang Province, China, and examined by histopathology and by electron microscopy.

Histopathological examination
Hepatopancreas, gill and stomach of both moribund and healthy mud crabs were fixed in Davidson's fixative for 24 hrs. The tissues were routinely processed, embedded, sectioned (5-7 μm) and stained with haematoxylin and eosin (H&E), according to the procedure of Weng et al. (2007). The tissues were examined under light microscopy.

Electron microscopy
After dissection, the heart, stomach, intestine and gill filaments were collected and fixed with 2.5% glutaraldehyde. Sample preparation for electron microscopy followed Yang and Wu (1992).

RESULTS

Necrotic lesions were easily observed in all five mud crabs with CD by staining with H&E (Fig.1A-C). Inclusion bodies, stained light red to deep red near the nucleus of infected cells, were also found in the gills of all specimens (Fig. 1A).

Transmission electron microscopy revealed that the reo-like viruses apparently caused vacuolar degeneration and necrosis of the infected cells in the connective tissues in heart, stomach, gills and intestines. Large numbers of virions with hexagonal shape were observed in the cytoplasm of enlarged gill (Fig. 2A) and intestine cells (Fig. 2B). The capsid measured approximately 60 nm from side to side, consisting of an electron-dense core surrounded by an electron-translucent zone (Fig. 2C), as well as free virions and nuclear structures in developing viruses (Fig. 2D). Crystal-like inclusion bodies produced by a large amount of mature viruses and viral intermediate structures were also present in the intestinal cells (Fig. 2B). However, the inclusion bodies could not be observed in the nucleus of intestinal cells. In stomach cells, a large amount of spherical viral particles aligned in a crystal lattice could be observed (Fig. 2E).

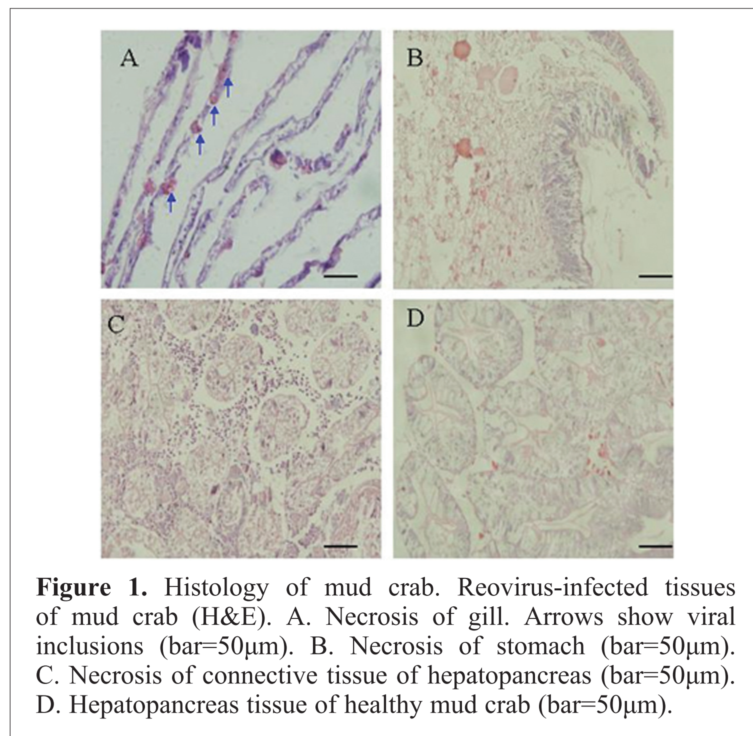

Figure 1. Histology of mud crab. Reovirus-infected tissues of mud crab (H&E). A. Necrosis of gill. Arrows show viral inclusions (bar=50μm). B. Necrosis of stomach (bar=50μm). C. Necrosis of connective tissue of hepatopancreas (bar=50μm). D. Hepatopancreas tissue of healthy mud crab (bar=50μm).

In some samples, gill tissue examined by TEM showed infection with mollicute-like organisms (Fig. 2F). The mollicute-like organisms, located in the cytoplasm of the host cell, varied in form from filate-shaped (3-5 μm) to flask-shaped with a head-like structure (2-3 μm). Another feature is the absence of a cell wall and they were only embedded by a plasma membrane.

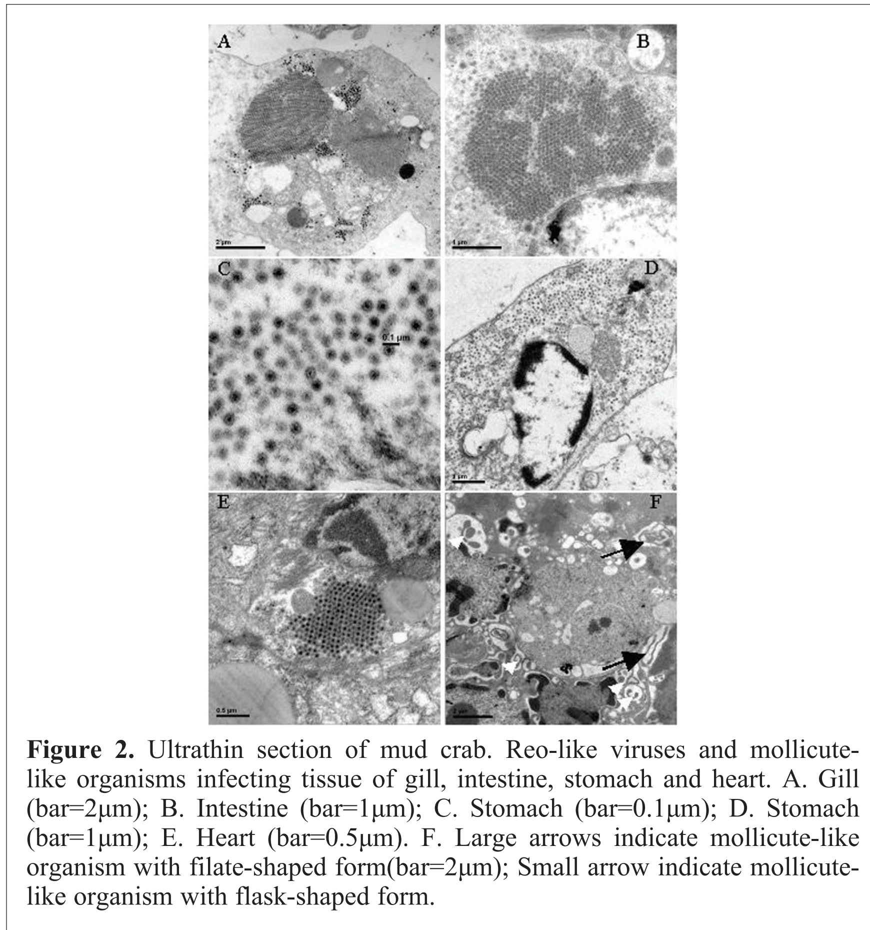

Figure 2. Ultrathin section of mud crab. Reo-like viruses and mollicute-like organisms infecting tissue of gill, intestine, stomach and heart. A. Gill (bar=2μm); B. Intestine (bar=1μm); C. Stomach (bar=0.1μm); D. Stomach (bar=1μm); E. Heart (bar=0.5μm). F. Large arrows indicate mollicute-like organism with filate-shaped form(bar=2μm); Small arrow indicate mollicute-like organism with flask-shaped form.

DISCUSSION

Since the viral isolation from *Macropipus depurator* L. (Vago *et al*., 1966), baculovirus, reovirus, parvovirus, rotavirus, and herpesvirus have been isolated from other species of crabs (Seidel *et al*., 1983; Mari and Bonami.,1988a; Mari and Bonami., 1988b; Montanie *et al*., 1993; Kanchanaphum et al., 1998; Sun and Guo., 1999., Gong *et al*., 2000; Zhang *et al*., 2002; Weng et al., 2007).

Several viruses lead to the diseases in *Scylla serrata,* such as white spot syndrome virus (WSSV) (Chen *et al*., 2000; Hameed *et al*., 2003), muscle necrosis virus (Song *et al*., 2003) and reovirus (Weng *et al*., 2007). The virus observed in the present study is similar to the members of the family Reoviridae, with respect to virus features, form and distribution, especially to the reovirus as reported by Weng *et al*. (2007). Based on these features, the virus is preliminarily identified as an aquatic reovirus. In order to show the difference to the reovirus found in *Eriocheir sinensis*, the present virus is termed temporarily as *Scylla serrata* reovirus (SsRV) on the basis of its host.

Previously studies have revealed that the reovirus was highly pathogenic and lead to various diseases in crabs, e.g. tremble disease in *Eriocheir sinensis* and sleep disease in *S. serrata* (Zhang *et al*., 2002). In terms of disease symptoms, it is proposed that the main pathogen of 'SD' in *Scylla serrata* may be SsRV, which was probably also the pathogen causing the sleeping disease in Fujiang Province and Guangdong Province. Further studies are required to confirm this conclusion.

Mycoplasmas of aquatic animals, especially those of Crustacea, have not been studied extensively. Krol *et al.* (1991) first reported a filamentous mollicute-like bacterium that appeared to be associated with necrotizing hepatopancreatitis lesions in cultured penaeid shrimp. Shortly after, Yang and Wu (1992) reported mollicute-like organisms that appeared to be associated with the gut-node disease, also in penaeid shrimps. Mollicute-like organisms would enter the perinuclear space, when the membrane of mollicute in the cytoplasm began to interact with the membranes in the perinuclear space. Moreover, the spherical nature and size of the mollicutes in the perinuclear space may result from nutritional factors or from environmental parameters (Yang and Wu, 1999). However, this described pathogenesis in shrimp still remains unclear. Isolation and culture of mycoplasma from penaeid was first reported by Ghadersohi and Owens (1999), in association with an epizootic of midcrop mortality syndrome in *Penaeus monodon*.

A tentative identification of the bacterium in *S. serrata* can be obtained based on morphology in preliminary electron micrographs. The intracellular microorganisms in gill cells of *S. serrata* lack a cell wall and are surrounded only by the plasma membrane. This is an indication that these microorganisms are mollicutes (Choi *et al*., 1996). The intracellular microorganisms are highly pleomorphic, and apparently an obligate intracellular pathogen. All of these facts are indications for mollicutes. The organism is now named 'gill mollicute-like organisms' (GMLO) to distinguish them from other mollicutes until their relationship is clarified.

We detected GMLO only in gill cells, not in other tissues. No evidence was found that gill cell necrosis in affected areas have been associated with MLO. Therefore, the virulence of mollicute-like organisms from *S. serrata* remains uncertain. Further studies are necessary to determine the identity of these bacteria and their pathogenesis in *S. serrata.*

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Status of shrimp diseases and advances in shrimp health management

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ABSTRACT

Disease has had a major impact on shrimp aquaculture since shrimp farming became a significant commercial entity in the 1970s. Diseases due to viruses, rickettsial-like bacteria, true bacteria, protozoa, and fungi have emerged as major diseases of farmed shrimp. Many of the diseases caused by bacteria, fungi and protozoans are now managed using improved culture practices, routine sanitation, and the use of probiotics and chemotherapeutics. However, the virus diseases have been far more problematic to manage and they have been responsible for the most costly epizootics. Because of their socioeconomic significance to shrimp farming, seven of the nine crustacean diseases currently listed (and two of three proposed for listing) by the World Organisation for Animal Health (= Office International des Epizooties or the OIE) are virus diseases of shrimp. The development and export of Specific Pathogen Free (SPF) stocks of *Penaeus vannamei* (the Pacific white shrimp) from the USA to the major shrimp farming countries of Latin America and SE Asia is cited by FAO as being the main contributor to the industry's recovery and subsequent expansion following the viral pandemics of the early 1990's. The development of SPF stocks of *P. vannamei* is the topic of this review.

Key words: shrimp diseases, shrimp health management, aquaculture, OIE, SPF

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INTRODUCTION

As recently as a decade ago, much of the world's production of farmed shrimp was directly or indirectly dependent on wild shrimp stocks for the "seed" stock used to populate its farms. In the Americas the most common practice was the collection and use of "wild seed" (postlarvae or PLs), while in Asia it was more typical to produce PLs from wild-sourced broodstock. While the practice of relying on the sea to provide its seed stock worked well for nearly two decades, the practice provided the industry with little protection from significant losses due to infectious diseases, such as those that were ultimately responsible for the major global shrimp disease pandemics that began around 1991-1992 (Flegel, 2006; Lightner, 2005; Lightner *et al*., 2009).

DOMESTICATED STOCKS - THE FIRST STEP TO SPF STOCKS

By the mid-1970s a number of penaeid shrimp research programs were developing culture systems and methods to close the life cycle of several penaeid shrimp species in captivity (Forester and Beard, 1974; Wickins and Beard, 1978; Salser *et al*., 1978; Aquacop, 1983; Moore and Brand, 1993). Some of these early research groups and institutions were successful in growing, maturing, mating, spawning, and producing progeny from founder shrimp stocks that had been reared for a full generation in captivity. Despite the early successes in developing captive breeding populations of penaeid shrimp at these various facilities, most of the shrimp farming industry remained dependent on the direct or indirect use of wild or captive-wild shrimp stocks for the PLs used to stocks its farms (Argue and Warren, 1999; Lightner, 2005; FAO, 2006). Nonetheless, during this period (~1980 to ~2000), the industry was experiencing much of its initial rapid growth. For example, before WSSV was introduced into Ecuador in 1999, more than 100,000 people were involved in the collection of wild postlarvae from the littoral zone for use in stocking Ecuador's more than 175,000 ha of shrimp ponds (Rosenberry, 2006).

The reasons for the dependence of the shrimp farming industry on wild shrimp stocks for seed were partially technical, but mostly economic. In most large shrimp farming regions of the Americas, the PL requirements were highly seasonal. Hatcheries (called "laboratories" in most of Latin America) were expensive to build, staff and run, and the seasonal requirements for PLs left them operating below capacity for lengthy periods each year. Further, wild PLs ("wild seed") could be obtained in large numbers seasonally (and often when needed most for seasonal stocking plans for farms) and for lower cost than hatchery produced PLs ("lab seed"). Another reason, with both economic and technical implications, was that the prevalence of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) in captive-wild *Penaeus vannamei* (the Pacific white shrimp) broodstock typically increased the longer the captive-wild stocks were held in maturation and/or hatchery facilities (Motte *et al*., 2003; Lightner *et al*., 2009). This made persistently IHHNV-infected captive-wild broodstock essentially worthless within 2-3 months of use as broodstock due to their declining performance. The use of *P. vannamei* broodstock with

high IHHNV prevalence resulted in poor survival of infected larvae and the production of poor quality PLs (Lightner and Redman, 1998; Motte *et al*., 2003). The surviving PLs ("lab seed" or "maturation seed") had a very high IHHNV prevalence relative to wild PLs. Ponds stocked with such PLs typically had poorer production levels due to the development of IHHNV-caused Runt-Deformity Syndrome (RDS) than did ponds stocked with wild PLs. Long term and increasing problems with IHHNV, and subsequently with Taura Syndrome (caused by TSV), and the arrival of White Spot Disease (caused by WSSV) in 1999 to Central America, Mexico, Ecuador and Peru resulted in rapid changes in shrimp farming strategies in the Americas. The yellow head and white spot disease pandemics in Asia that began about 1992 and the paucity of domesticated SPF stocks of Asian penaeid shrimp species, forced the worlds' shrimp farming industries to change how shrimp are farmed (Lightner, 2005; FAO, 2006; Lightner *et al*., 2009).

FAO (2006) credits the development and export from producers in the USA of SPF (specific pathogen-free) *P. vannamei* and *Penaeus stylirostris* (the Pacific Blue Shrimp) for this paradigm shift in shrimp farming. The FAO report goes on to comment that while the export of SPF shrimp stocks from the USA to Asia and elsewhere in the world may not have been significant in their quantity or total value, their impact has been considerable on both the total quantity of shrimp produced and on global shrimp pricing. The FAO report concludes that without the import and use of USA-produced SPF shrimp stocks it is arguable if Asia's major shrimp producing countries could have recovered from disease outbreaks and the severe shortage of healthy wild-caught broodstock of native penaeids, much less grown to achieve the record levels of production that currently characterize the Asian shrimp farming sector (FAO, 2006).

In the wake of the extraordinary losses that occurred as a result of the viral pandemics of the early 1990's, the industry began to mature into a much more sustainable, technology-based industry. The industry has largely recovered from the major viral pandemics and it has begun a new phase of rapid growth (FAO, 2006). The adoption of new shrimp farming technologies and the abandonment of practices which posed high disease risks have contributed to the industry's recovery and current expansion. Among the most notable changes in culture practices has been the shift of the industry away from using wild stocks for seed production to the use of domesticated stocks. This has been a consequence of the ever increasing incidence in wild shrimp stocks of diseases like WSSV, IHHNV, and other significant diseases that negatively affect broodstock or their progeny (Lightner, 2005; Flegel, 2006). This has made the collection of wild postlarvae (PLs) and wild adult broodstock for the production of PLs for use as seed stock, a risky practice. With the declining dependence of the industry on wild stocks in Asia and in the Americas, the use of domesticated lines of specific pathogen-free (SPF) *P. vannamei* recently surpassed *Penaeus monodon* (the Giant Black Tiger Shrimp) as the dominant farmed shrimp species in Asia. This paradigm switch in the species being farmed occurred within 5 years after the first SPF *P. vannamei* stocks were introduced in quantity to Asia. The use of SPF *P. vannamei* has led to improved production and predictable crops virtually everywhere that was once dominated

by the culture of *Penaeus chinensis* (the Chinese White Shrimp) or *P. monodon*. Hence, the most significant single advance in shrimp health management was perhaps the development of domesticated lines of SPF *P. vannamei.*

DEVELOPMENT OF SPF STOCKS

The term "SPF" was in widespread use in large number of terrestrial animal, aquatic animal and plant agriculture industries prior to its being applied to shrimp aquaculture (Wyban, 1992; Wyban *et al*., 1992; Lotz, 1992; Carr *et al*., 1994; Pruder *et al*., 1995; Lotz, 1997a, 1997b; Moss and Moss, 2009). SPF culture practices were commonplace in the poultry, swine and trout producing industries for many years and are still used as a way to avoid disease in otherwise susceptible stocks, when no other means of therapy or prevention (without the increased costs or other problems associated with vaccination) are available (Zavala, 1999). The application of the SPF concept to shrimp farming was a relatively recent event and it occurred well after the technologies had been developed that were necessary to close the life cycle of the penaeid shrimp in the laboratory and begin the process of producing domesticated breeding lines of penaeid shrimp. The development of SPF shrimp stocks became possible with the simultaneous development of the necessary infrastructure, in terms of biosecurity, diagnostic methods and trained personnel, to successfully select founder populations of candidate SPF stocks from wild or cultured stocks and domesticate those stocks, following the ICES Guidelines (ICES, 1995), in the absence of specific disease agents under biosecure conditions (Moss and Pruder, 1999; Bullis and Pruder, 1999; Lightner, 2003a, 2003b, 2005; Lee and O'Bryen, 2003; Scarfe *et al*., 2006; Lightner *et al*., 2009; Moss and Moss, 2009).

The first SPF stocks developed by the U.S. Marine Shrimp Farming Program (USMSFP) were developed in the spirit of the ICES Code (The International Council for the Exploration of the Sea; Code of Practice to Reduce the Risks of Adverse Effects Arising from the Introduction on Non-indigenous Marine Species - Bartley *et al*., 1996) (Table 1) (Wyban *et a*l., 1992; Carr *et al*., 1994; Pruder *et al*., 1995). The determination of which specific pathogens the selected stocks of candidate SPF shrimp were to be free of was based on a working list of pathogenic, diagnosable, and excludable pathogens. The SPF list necessarily changed over time as new diseases, such as those due to WSSV, TSV, IMNV and others, emerged and caused or showed the potential to cause serious pandemics (Lightner *et al*., 2009). The most current working list for the U.S. Marine Shrimp Farming Program includes 10 viruses or virus groups (WSSV, the YHV group, TSV, IHHNV, hepatopancreatic parvovirus (HPV), *Baculovirus penaei* (BP), monodon baculovirus (MBV), baculoviral mid-gut gland necrosis (BMN), and infectious myonecrosis (IMNV) and *Penaeus vannamei* nodavirus (*Pv*NV)), certain classes of parasitic protozoa (microsporidians, haplospordians, and gregarines), and the bacterial agent of necrotizing hepatopancreatitis, or NHP (Table 2). The USMSFP list closely approximates the OIE listed diseases of penaeid shrimp, with all seven of the currently OIE-listed virus diseases of shrimp (and one viral and one bacterial disease being considered for listing) being on the USMSFP list.

Table 1. Recommended steps in the ICES guidelines for risk reduction in aquatic species introductions (modified from Sindermann, 1988, 1990 and Lightner, 2005).

Original ICES Guidelines	**Adapted to SPF shrimp development**
1. Conduct comprehensive disease study in native habitat.	1. Identify stock of interest (i.e., cultured or wild).
2. Transfer {founder stock} system in recipient area.	2. Evaluate stock's health/disease history.
3. Maintain and study closed system population.	3. Acquire and test samples for specific listed pathogens (SLPs) and pests.
4. Develop broodstock in closed system.	4. Import and quarantine founder (F0) population; monitor F0 stock.
5. Grow isolated F1 individuals; destroy original introductions.	5. Produce F1 generation from F0 stock.
6. Introduce small lots to natural waters - continue disease study.	6. Culture F1 stock through critical stage(s); monitor general health and test for SLPs.
	7. If SLPs, pests, other significant pathologies are not detected, F-1 stock may be defined as SPF and released from quarantine.

To begin the process of developing an SPF stock a candidate wild or farmed stock of interest was identified (Fig. 1). If available, samples of the stock were taken and tested using the most appropriate diagnostic and pathogen detection methods available for the specific pathogens of concern. If none were found, a founder population (F_0) of the "candidate SPF" stock was acquired and reared in primary quarantine. During primary quarantine, the F_0 stock was monitored for signs of disease, sampled, and tested periodically for specific pathogens. If any pathogens of concern were detected, the stock was destroyed. Those stocks that tested negative for pathogens of concern through primary quarantine (which ran from 30 days to as much as one year for some stocks) were moved to a separate secondary quarantine facility for maturation, selection, mating, and production of a second (F_1) generation. The F_1 stocks were maintained in quarantine for further testing for specific pathogens of concern. Those that tested negative were designated as SPF, and used to produce domesticated lines of SPF shrimp (Moss *et al*., 2003; Lightner, 2005; Lightner *et al*., 2009) (Fig. 1). With this practice, the definition for SPF shrimp stocks produced by the USMSFP meant that the stock of interest had at least two years of documented historical freedom of the disease agents listed on its working list of specific pathogens, that the stock has been cultured in biosecure facilities, and that the stock was either cultured under conditions where the listed disease agents would have produced recognizable disease if any were present and/or that the stock has been subjected to routine surveillance and testing for the listed pathogens (Lightner, 2005; Lightner *et al*., 2009) . Those pathogens on the USMSFP SPF list have also met

Table 2. Current U.S. Marine Shrimp Farming Consortium (USMSFC) working list of "specific" and excludable pathogens of American penaeids and Asian penaeids for 2008-2009 (adapted from and Lightner *et al*., 2009).

Pathogen Type	Pathogen/Pathogen Group	Pathogen Category[a]
Viruses	* WSSV - white spot syndrome virus (Nimaviridae, new family)[b]	C-1
	* YHV, GAV, LOV - the Oka viruses (Roniviridae, new family)[b]	C-1
	* TSV - (Dicistroviridae, new family in the picornavirus superfamily)[b]	C-1
	* BP[c] - an occluded enteric baculovirus	C-2
	* MBV[c] - an occluded enteric baculovirus	C-2
	BMN[c] - a nonoccluded enteric baculo-like virus	C-2
	* IHHNV - a systemic parvovirus	C-1
	SMV - an enteric parvovirus	C-2
	** HPV - enteric parvoviruses	C-2
	* IMNV - Infectious myonecrosis virus (putative totivirus)	C-1,2
	PvNV - *Penaeus vannamei* nodavirus	C-2
Procaryotes	** NHP-bacterium - Alpha proteobacteria	C-2
Protozoa	Microsporidians	C-2
	Haplosporidians	C-2
	Gregarines	C-3

* OIE listed pathogen (OIE 2008).

** Listed as "under study" (OIE 2008).

[a] Pathogen category (modified from Lotz *et al*., 1995), with C-1 pathogens defined as excludable pathogens that can potentially cause catastrophic losses in one or more American penaeid species; C-2 pathogens are serious, potentially excludable; and C-3 pathogens have minimal effects, but may be excluded from breeding centers, hatcheries, and some types of farms.

[b] Mayo, 2002a, 2002b.

[c] The 1995 Committee report on virus taxonomy (Murphy *et al*., 1995) removed crustacean baculoviruses from the *Baculoviridae* and assigned them to a position of unknown taxonomic position. Nonetheless, BP, MBV, and BMN are most like members of the *Baculoviridae* (Faquet *et al*., 2005), and for practicality, they are listed here as baculoviruses.

certain criteria including: (a) the pathogen(s) must be excludable; (b) adequate diagnostic and pathogen detection methods are available; and (c) that the pathogen(s) poses significant threat of causing disease and production losses, criteria which are also among those required for disease listing by the OIE.

After the criteria set forth in the ICES Code had been met and a particular stock is declared SPF of specified diseases/pathogens, maintenance of SPF status required that the domesticated SPF stocks be the subject of a routine surveillance program. To be functional,

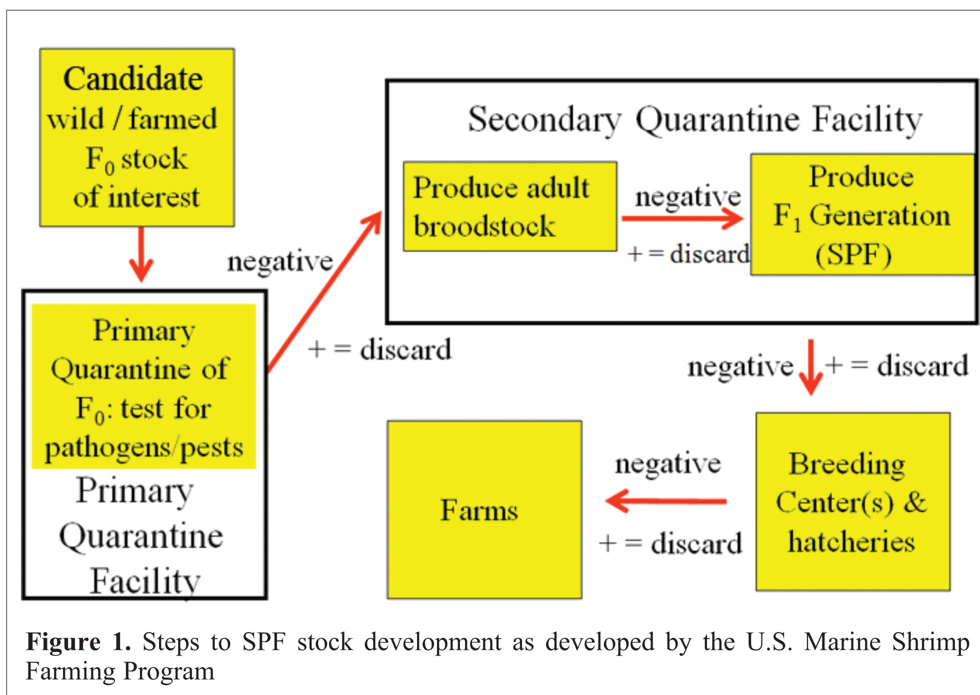

Figure 1. Steps to SPF stock development as developed by the U.S. Marine Shrimp Farming Program

an SPF breeding program must have a surveillance program with both regularly scheduled targeted and general (passive) surveillance components. Molecular diagnostic methods have become as important as classical methods (such as routine histopathology and microbiology) to the shrimp culture industry in recent years, and are especially applicable to routine surveillance programs that are necessary to support claims of disease freedom (such as with SPF stocks) and to monitor shrimp stocks in farms (Subasinghe *et al*., 2004; OIE, 2006; OIE, 2008; Lightner, 2005; Lightner *et al*., 2009).

Because the term SPF is poorly understood and often misused, the term "high health" has also been borrowed from other animal producing industries for use with shrimp to designate shrimp stocks that were developed as SPF, and which may be free of infection by specific disease agents, but which are no longer contained within a designated biosecure SPF facility (Pruder *et al*., 1995). SPF and high health stocks of *P. vannamei* were introduced by the USMSFP and used successfully in U.S. shrimp farms in the mid-1990's and this resulted in nearly doubling the production per crop that had been previously obtained at the same farms in previous years when the farms cultured non-selected lines of *P. vannamei*, which in previous crops, had been persistently affected by RDS due to chronic infection by IHHNV (Wyban, 1992; Pruder *et al*., 1995; Lotz *et al*., 1995; Moss *et al*., 2003).

Beginning in 1999, significant quantities of SPF *P. vannamei* had been introduced into East Asia and found to perform well. By 2006, nearly 3 million metric tonnes of the marine penaeid shrimp were being produced from farms and these shrimp accounted for almost half of the world's total shrimp supply (FAO, 2006). More than half (~57%) of that 3 million

pounds was made up of *P. vannamei.* Ironically, more *P. vannamei* were farmed in 2006 in Asia, where the species was introduced, than in the Americas where it is native. FAO in its 2006 publication, "State of world Aquaculture," credited the development and export (from producers in the USA) of SPF *P. vannamei* for this paradigm shift in shrimp farming. The use of SPF *P. vannamei* has led to less disease, improved survival and predictable crops virtually everywhere that was previously dominated by *P. chinensis* and *P. monodon* (FAO, 2006).

SPECIFIC PATHOGEN RESISTANT (SPR) SHRIMP STOCKS

After domesticated SPF stocks were developed some SPF/SPR stocks were successfully developed and applied to management of certain shrimp virus diseases, specifically Taura Syndrome (TS) and Infectious Hypodermal and Hematopoietic Necrosis (IHHN) in some locations (Lightner and Redman, 1998; Fegan and Clifford, 2001; Moss and Moss, 2009; Lightner *et al*., 2009). While unselected stocks of *P. vannamei* have a high degree of resistance to IHHN, the degree of resistance has been improved in some locations by the breeding of selected individuals which show greater resistance to IHHN and RDS (a chronic form of IHHN disease in *P. vannamei*) than unselected stocks (Fegan and Clifford, 2001; Lightner *et al*., 2009). From some of these stocks, IHHNV-free founder stocks have been developed, and these have the advantage of being SPR for IHHN disease, but also being SPF for IHHNV and other diseases.

SPR stocks have been most successfully used in culture regions where TSV, IHHNV or both diseases are enzootic in wild penaeid shrimp stocks or where the viruses are readily transmitted within or between farms. "SPR-43" stocks of *P. stylirostris* were the first SPR stocks developed and they are the primary stocks currently farmed in New Caledonia and French Polynesia. Before WSSV reached Mexico in 1999, SPR stocks of *L. stylirostris* (SPR-43 developed in French Polynesia and Super Shrimp developed in Venezuela) were the dominant shrimp stocks cultured in Mexico and they accounted for nearly 80% of the farmed shrimp produced in Mexico in 1998.

The SPR-43 and Super Shrimp lines of *P. stylirostris* were developed over time in captive stocks of *P. stylirostris* by breeding survivors that were persistently infected with IHHNV. Breeding survivors to survivors eventually resulted in continuous domesticated lines of *P. stylirostris* with a high degree of resistance to IHHN disease, despite being persistently infected with the virus. Some lines of Super Shrimp were found in laboratory challenge studies with IHHNV to be resistant even to infection and to quickly clear the virus after challenge (Tang *et al*., 2010). The Super Shrimp line was developed using the same strategy as was used for SPR-43. Super Shrimp came from breeding survivors of stocks initially imported into Venezuela from Panama. When the stocks were introduced into Mexico in ~1994 1995, the SPR-43 stock was at about its 18th generation in captivity and the Super Shrimp lines were at about their 16th generation (Fegan and Clifford, 2001; Lightner, 2005; Lightner *et al*., 2009).

More recently, SPR stocks of SPF *P. vannamei* were developed by the USMSFP by selectively breeding for TSV resistance. In screening the various lines of SPF, domesticated *P. vannamei* reared by the program for TSV resistance in controlled laboratory challenge trials, certain geographic stocks were found to present better survival than stocks whose founders were derived from other geographic regions. These were selectively bred for multiple generations, and with each generation checked experimentally for TSV resistance. Such SPR stocks of *P. vannamei* (which are also SPF for all OIE and USMSFP listed diseases of penaeid shrimp - Table 2), are currently commercially available from most broodstock suppliers in the USA. Most of the TSV resistant SPF/SPR stocks currently available have documented survival rates of 80 to 100% in laboratory challenge studies with four TSV isolates that represent the main geographic genotypes/biotypes of this virus. The Belize genotype of TSV is the most virulent biotype of the four known TSV genotypes (Moss and Moss, 2009; Lightner *et al*., 2009). Fortunately, selection for resistance to the reference TSV genotype/biotype (obtained from Ecuador and Hawaii in 1994) has been found to work well for all TSV genotypes/biotypes to date (Lightner *et al*., 2009).

FUTURE OF SPF STOCK DEVELOPMENT

Following the model for SPF stock development used by the USMSFP for the development of SPF *P. vannamei*, companies in Asia, Madagascar and the USA have developed some SPF *P. monodon* and *Penaeus chinensis* stocks and others are developing SPF *P. indicus* (Hennig *et al*., 2005; Pantoja *et al*., 2005; Lightner *et al*., 2009). Despite the significant challenges posed by disease, the shrimp farming industry has responded to the challenges posed and it has developed methods to manage disease and mature into a more sustainable industry. Adoption of the SPF concept in the domestication of *P. vannamei* and development of the species for aquaculture was among the milestones that have led to the industry's current explosive growth and apparent sustainability (FAO, 2006). As it has in other meat producing industries, the development of SPF stocks of *P. vannamei, P. stylirostris, P. monodon*, and other penaeid species has become central to the sustainability of modern shrimp farming. Further development through selective breeding of SPF stocks for disease resistance and other desired characteristics is very likely to be a principle characteristic of the industry for the indefinite future.

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A review of the strategies evolved by WSSV to thwart host responses to infection and ensure successful virus replication in cells - apoptosis and anti-apoptosis strategies

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ABSTRACT

During virus infection, premature cell death (*apoptosis*) blocks production of progeny virus. Some viruses have evolved ways to block apoptosis to ensure their replication, and some viruses can even induce apoptosis to assist their own dissemination. White spot syndrome virus (WSSV) infection induces apoptosis in shrimp, and the infected tissues display the characteristic signs of apoptosis, *i.e.* nuclear disassembly, fragmentation of DNA into a ladder, and increased caspase-3 activity. WSSV-induced apoptosis occurs in bystander, non-infected cells, whereas the infected cells are non-apoptotic. Although the factors that induce apoptosis are currently unknown, it has become clear that shrimps use apoptosis as a protective response to prevent the spread of WSSV. To counter this, WSSV is now known to produce at least two anti-apoptosis proteins that block apoptosis and thus facilitate viral multiplication.

Key words: apoptosis, anti-apoptosis, WSSV, shrimp

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WSSV-INDUCED APOPTOSIS: THE BEGINNING

Henderson and Stuck (1999) were first to report that apoptosis levels were increased in moribund *Penaeus* (*Litopenaeus*) *vannamei* shrimp infected with WSSV. In a subsequent report (Sahtout *et al*., 2001), *Penaeus monodon* shrimp from commercial cultivation ponds were diagnosed first through histological examinations and then PCR, and some of them were found to be WSSV-positive. All of the shrimp were then examined with TUNEL and agarose gel electrophoresis of DNA to detect the occurrence of apoptosis. Sahtout *et al*. (2001) found that in grossly normal shrimp negative for WSSV by PCR, no TUNEL-positive cells were detected. Conversely, shrimp showing gross signs of WSSV infection had TUNEL-positive cells, and the number of TUNEL-positive cells was proportionally related to the severity of the WSSV infection. Lightly infected shrimp (2-step PCR positive) gave mean counts of 16 ± 8% TUNEL-positive cells, whereas heavily infected shrimp (1-step PCR positive) had mean counts of 40 ± 7% TUNEL-positive cells. Among the examined tissues, abdominal epithelium had the highest level of TUNEL-positive cells, followed by the stomach epithelium, hepatopancreatic interstitial cells, gills and muscle. Another indication of apoptosis was that agarose gel analysis of DNA extracts from the WSSV-infected shrimp showed DNA smears or ladders. The authors suggested that apoptosis induced by WSSV in numerous tissues/organs might contribute to shrimp death.

Although the above study showed that shrimp naturally infected with WSSV contained numerous apoptotic cells, there remained the possibility that the observed apoptosis may have been due to other viral pathogens. Viral co-infection in shrimp is quite common, and it is hard to tell whether apoptosis in naturally infected shrimp is in fact induced by WSSV or by another viral pathogen. The possibility of this kind of confounding factor is especially problematic when there is no information about how long the shrimp have been infected or how soon after infection the apoptosis occurred. Two years after the Sahtout *et al*. (2001) study, Wongprasert *et al*. (2003) experimentally infected *P. monodon* shrimp and performed a time course study to evaluate apoptosis levels in various tissues. DAPI staining showed that hemocytes with condensed and fragmented nuclei, *i.e.* apoptotic cells, were first detected at 24 hpi, and their numbers increased significantly thereafter. At 60 hpi, 20% of the hemocytes were apoptotic. For other tissues, the subcuticular epithelium displayed the highest level of TUNEL-positive cells (10% at 36 hpi), and these were detected as early as 6 hpi. In gills and hematopoietic tissues, TUNEL-positive cells were first detected at 24 hpi and their numbers significantly increased by 36 hpi. The occurrence of apoptosis in various tissues was further confirmed by TEM. More importantly, TEM showed that apoptotic features such as chromatin margination and nuclear condensation and fragmentation only occurred in cells that contained no virions; in cells where WSSV virions were present, no signs of apoptosis were observed. This study also reported that caspase-3 activity of the subcuticular epithelium was 6-fold higher in WSSV-infected shrimp.

Both Sahtout *et al*. (2001) and Wongprasert *et al*. (2003) reported that some cells with hypertrophied nuclei (*i.e.* WSSV-infected cells), were TUNEL-positive. Under TEM, Wongpraset *et al* . 2003) confirmed that the chromatin in such cells was not condensed and

did not break up into apoptotic bodies. They proposed that either these cells followed a non-typical apoptotic pathway or that the fragmentation of the DNA was caused by an activity of WSSV that was unrelated to apoptosis. It now appears quite possible that WSSV intentionally degrades host DNA for its own benefit, as two WSSV proteins have recently been implicated in DNA degradation: a nuclease protein (Li *et al*., 2005) and a novel multifunctional protein called ICP11 are both able to promote DNA degradation (Wang *et al*., 2008).

The apoptosis response in WSSV-infected *P. vannamei* shrimp has also been investigated. The observations reported by (Granja *et al.,* 2003) were in general agreement with those for infected *P. monodon*. In both species, apoptosis occurs in WSSV-infected tissues, and the incidence of apoptotic cells increases with time. One discrepancy noted by Granja *et al.* (2003) was that their TUNEL-positive cells did not have hypertrophied nuclei, although this is a characteristic that would be expected in WSSV-infected cells. Granja *et al.* (2003) considered this unexpected result to be accurate because they used the less sensitive colorimetric TUNEL method, and this allowed the morphology of the cells to be observed under the light microscope, thus reducing the risk of misinterpretation and false positives.

Wu and Muroga (2004) used TUNEL to investigate apoptosis responses in the lymphoid organ (LO) and stomach epithelium of WSSV-infected kuruma shrimp. The shrimp were experimentally infected with high or low doses of WSSV. In LO, high infection doses produced higher numbers of apoptotic cells while the incidence of apoptotic cells decreased with the progress of WSSV infection. The highest number of apoptotic cells was observed at 12hpi (4.5%±3.8) and then decreased thereafter. Curiously, there were only very few TUNEL-positive cells in the stomach epithelium, regardless of whether the infection dose was high or low. Compared to *P. monodon* and *P. vannamei*, Kuruma shrimp (*Penaeus japonicus*) therefore seem to have different apoptosis responses toward WSSV infection. In this study, tissue sections adjacent to those used for TUNEL staining were also subjected to *in situ* hybridization (ISH) with a WSSV probe, and comparison of these neighboring sections showed that TUNEL-positive cells were ISH-negative, confirming that cells infected with WSSV were non-apoptotic, and that apoptotic cells had no WSSV.

A comparative study in hemocytes for WSSV-infected *P. monodon* and *Penaeus indicus* using flow cytometry showed that the number of apoptotic hemocytes was higher in *P. indicus* than in *P. monodon* (60% vs. 20%), and that in both species, the number of apoptotic hemocytes increased with time (Sahul Hameed *et al*., 2006). This suggested that *P. indicus* hemocytes are more sensitive to WSSV infection than *P. monodon* hemocytes.

There are relatively few studies of WSSV-induced apoptosis in crustaceans other than penaeid shrimp. In crayfish, WSSV infection induces hemocytes to undergo apoptosis, and the percentage of apoptotic hemocytes is quite low: 1.5% in crayfish (Jiravanichpaisal *et al*., 2006) vs. 20% in shrimp (Wongprasert *et al*., 2003).

Overall, based on these studies, we can conclude that WSSV infection induces apoptosis in crustaceans, and that the incidence of apoptosis is both species-specific and tissue-

specific, and is related to the severity of WSSV infection. Further, the apoptosis occurs only in uninfected by-stander cells, whereas WSSV-infected cells are non-apoptotic.

WSSV ANTI-APOPTOSIS GENES

There is evidence that at least two WSSV proteins are involved in anti-apoptosis: ORF390 (we tentatively called this protein WSSV anti-apoptosis protein-1, AAP-1, and we use this name throughout this paper; Wang *et al*., 2004) and WSSV222 (He *et al*., 2006). The gene encoding AAP-1 was identified through a genetic complementation assay for rescuing the multiplication of a mutant insect virus in host cells. Figure 1 shows a schematic summary of this assay.

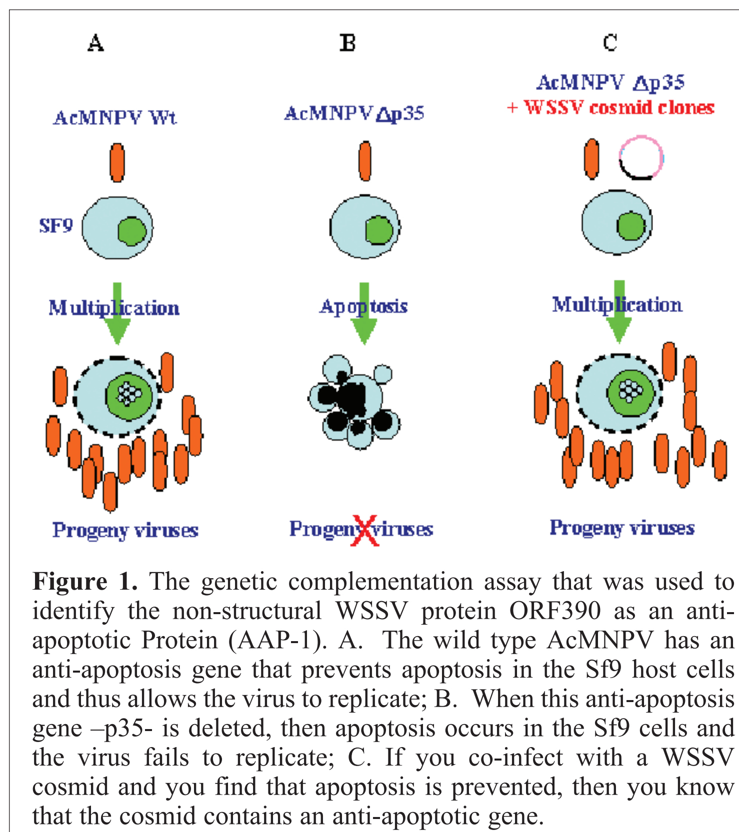

Figure 1. The genetic complementation assay that was used to identify the non-structural WSSV protein ORF390 as an anti-apoptotic Protein (AAP-1). A. The wild type AcMNPV has an anti-apoptosis gene that prevents apoptosis in the Sf9 host cells and thus allows the virus to replicate; B. When this anti-apoptosis gene –p35- is deleted, then apoptosis occurs in the Sf9 cells and the virus fails to replicate; C. If you co-infect with a WSSV cosmid and you find that apoptosis is prevented, then you know that the cosmid contains an anti-apoptotic gene.

Briefly, the insect virus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), contains an anti-apoptosis gene, *p35*, that is essential for the multiplication of AcMNPV in SF cells. Mutation/deletion of *p35* induces apoptosis during AcMNPV infection, and this severely impairs the production of progeny virus. However, if multiplication of the *p35*-mutated AcMNPV in SF cells is restored by overexpresssion of an unknown anti-apoptosis protein, then it follows that the candidate must be capable of blocking apoptosis. Based on this strategy, a WSSV cosmid library was constructed and the cosmid vectors were

co-transfected with *p35*-mutated AcMNPV DNA into SF cells to isolate WSSV genes that could restore the multiplication of *p35*-mutated AcMNPV. By repeating the experiments many times, WSSV anti-apoptosis protein AAP-1 was finally identified, and it appeared to function like AcMNPV P35 protein (Wang *et al*., 2004). In insect cells this protein blocked apoptosis induced by Actinomcyin D, *Drosophila* pro-apoptosis protein Rpr, and *P. monodon* caspase3 (Pm caspase3) (Wang *et al*., 2004; Leu *et al*., 2008a,b). The fact that AAP-1 could block apoptosis induced by different stimuli, suggests that it is acting at a conserved, central point in the apoptosis pathway. We recently (Leu *et al*., 2008b) showed that AAP-1 was both a substrate and inhibitor for Pm caspase-3, and that only cleaved APP1 directly bound to Pm caspase-3. Accordingly, we hypothesized that after AAP-1 is recognized and cleaved by caspase, it functions by directly binding to the active site of caspase, preventing the caspase from further accessing other substrates. AAP-1 contains a caspase-3 cleavage site, which seems to be responsible for both the cleavage by and also the inhibition of caspase. Our unpublished data shows that point mutation at this caspase-3 cleavage site abolishes the anti-apoptosis activity of AAP-1.

Another anti-apoptosis protein is WSSV222. This is one of the four WSSV proteins (the other three are WSV199, WSV249 and WSV403) that are predicted to have a RING-H2 finger motif. This motif is involved in ubiquitin-conjugating enzyme (E2)-dependent ubiquitination, and many proteins containing a RING finger play a key role in the ubiquitination pathway. He *et al*. (2006) first showed that WSSV222 had RING-H2-dependent E3 ligase activity *in vitro* and then used a yeast two-hybrid assay to identify its interaction partner, the tumor suppressor-like protein (TSL). They further showed that WSSV222 could ubiquitinate the TSL, and that the ubiquitinated TSL then underwent proteasome-dependent degradation in a mammalian cell line and in a primary shrimp tissue culture of shrimp cells infected with WSSV. Since transient expression of TSL in BHK mammalian cells caused apoptosis, and this could be blocked by coexpressing WSSV222 in the same cells, they hypothesized that in WSSV-infected shrimp, WSSV222 functions as an anti-apoptosis protein through ubiquitin-mediated degradation of TSL. By preventing apoptosis, WSSV222 thus facilitates the propagation of WSSV.

Although both AAP-1 and WSSV222 exhibit anti-apoptosis activity, they apparently act through different mechanisms. AAP-1 is a direct caspase inhibitor and it can evidently block apoptosis triggered by different stimuli, whereas WSSV222 only blocks apoptosis triggered by a specific but currently unknown protein(s) through ubiquitin-mediated degradation. We also note that WSSV222 is an early protein while AAP-1 is a late protein. Thus WSSV evidently deploys different anti-apoptotic strategies at different stages of infection.

INHIBITION OF APOPTOSIS: PROS AND CONS FOR THE HOST AND VIRUS

As WSSV uses at least two different anti-apoptosis proteins and at least two different mechanisms to suppress the occurrence of apoptosis in infected cells, it can be inferred that the induced apoptosis must be an anti-viral response that is deleterious to the multiplication

of WSSV. But to what extent is apoptosis beneficial or detrimental to the shrimp host itself? At low levels, apoptosis can remove the infected cells without harm to the shrimp host, but when extensive apoptosis occurs, dysfunction or failure of the affected tissues/organs might lead to the demise of the shrimp. Therefore, apoptosis is a double-edged sword. Two studies have explored this question by manipulating the expression of caspase. Caspases are a group of structurally related cysteine proteases that play important role in apoptosis. Based on their functions and structural features, caspases are classified into two different groups, initiator and effector caspases. At least three different caspases have been cloned from penaeid shrimp: two effectors and one initiator (Phongdara *et al*., 2006; Leu *et al*., 2008b; Wang *et al*., 2008). An initiator caspase gene, *Pjcaspase*, was identified in *Masupenaeus japonicus* (Wang *et al*., 2008). This gene was upregulated in survivors of WSSV-challenged shrimp. To investigate the importance of this initiator caspase gene against WSSV infection, the authors silenced this gene through siRNA. In WSSV-infected shrimp, when *Pjcaspase* was down regulated, caspase-3 activity and the number of apoptotic hemocytes were both decreased and, conversely, the number of copies of WSSV in the hemolymph was increased. This paper demonstrated that apoptosis could indeed be regarded as a host anti-viral defense system, and that the down-regulation of an initiator caspase gene favored the multiplication of WSSV. However, this study did not investigate the extent to which the WSSV-induced apoptosis may have contributed to the death of the shrimp.

In contrast to the above report for an initiator caspase, when an effector caspase gene was silenced in WSSV-infected shrimp, the opposite result was produced. Rijiravanich *et al*. (2008) used dsRNA to suppress the expression of *P. vannamei* caspase-3 genes, and when the shrimp were challenged with a high dose of WSSV, there was no observed effect on shrimp mortality. On the other hand, when challenged with a low dose of WSSV, the caspase-3 dsRNA injection group exhibited a lower mortality (27%) compared to the nonspecific dsRNA injection group (52%). The authors therefore concluded that silencing this caspase-3 gene partially protected the shrimp against death induced by WSSV infection. However, the authors did not report whether the number of apoptotic cells, the caspase-3 activity or the number of WSSV copies were changed in the caspase-3 silenced shrimp. Currently, although the dsRNA-based gene silencing technique is widely used in shrimp, the underlying mechanism remains largely unknown. Even so, it is known that dsRNA produces non-specific anti-viral activity (Robalino *et al*., 2005; 2006), so it is quite possible that the protection effect of caspase-3 dsRNA was due to such non-specific activity. We also note that, as would be expected, the control dsRNA group showed increased protection against WSSV infection when compared to the WSSV-infected shrimp with no dsRNA injection, which had a mortality of 79% (Rijiravanich *et al.,* 2008).

The administration of an apoptosis inhibitor to shrimp is a more direct method to evaluate the importance of apoptosis to WSSV infection and shrimp death. Wang and Zhang (2008) experimentally injected shrimp with the apoptosis inhibitor z-VAD-FMK, and then infected the shrimp with WSSV. They found that the WSSV-infected shrimp with z-VAD-FMK had a lower number of apoptotic hemocytes, increased WSSV copies, and increased mortality.

Since the inhibition of apoptosis resulted in a reduction of the number of surviving shrimp, the authors concluded that the net effect of apoptosis is to protect the infected shrimp from demise, rather than to cause the shrimp to die.

The notion that apoptosis protects the WSSV-infected shrimp from mortality coincides with a study by Granja *et al*. (2003), which investigated the mechanism involved in the beneficial effect of hyperthermia for WSSV-infected *P. vannamei* shrimp. Hyperthermia (32°C) increased the survival of WSSV-infected shrimp. Compared to shrimp kept at normal temperature (25°C), *in situ* hybridization showed a decrease in the number of WSSV-positive cells. Conversely, the incidence of apoptotic cells increased under hyperthermic conditions. Granja *et al*. (2003) suggested that at 32°C, the increased incidence of apoptosis might be the mechanism by which the shrimp were able to avoid death.

CONCLUSION

Some progress has been made toward the understanding of WSSV's anti-apoptosis mechanisms However, it remains unclear how WSSV induces apoptosis in by-stander cells. As for the host, compared to other model organisms, the study of the mechanisms and molecules involved in shrimp apoptosis is still in its infancy. Studies of vertebrate/virus interactions reveal that host cells have many cellular sensors that can detect the activities of an infecting virus and then transmit the pro-apoptosis signals to initiate cell death. These cellular sensors include death receptors, protein kinase R, mitochondrial membrane potential, p53 and the endoplasmic reticulum (Everett and McFadden, 1999). We hypothesize that detecting sensors should exist in shrimp cells as well. During the course of WSSV infection, these sensors could transmit signals, such as the binding of WSSV to its receptors, the transcription of WSSV genes or the replication of the WSSV genome, to trigger apoptosis. Before or during the execution of the apoptosis program, WSSV anti-apoptosis proteins such as WSSV222 and AAP-1 would begin to function and block further progress of apoptosis, so that WSSV could complete its replication cycle. In the case of apoptotic by-stander, cells, we speculate that these cells might have actually been infected with WSSV, but that for some reason either the two anti-apoptosis proteins do not function well or the pro-apoptotic signals are too strong to be blocked; consequently, apoptosis begins and the multiplication of WSSV is prevented.

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Effects of extensive and intensive shrimp farming on the genetic composition of white spot syndrome virus populations

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ABSTRACT

White spot syndrome virus (WSSV) has a major negative impact on shrimp farming and industry. There are many different shrimp farming practices, and these may affect the genotypic composition of WSSV populations and possibly the virulence of the virus. Here we investigated whether extensive and intensive farming practices (1) result in selection of WSSV genotypes, and (2) affect genotypic composition over time in WSSV populations. WSSV samples were collected from Vietnamese farms on various sites over a period of several years and the samples were then genotyped. We found no significant effect of farm practice on the genotypic composition of WSSV populations. On the other hand, we did find an effect of farm practice on change over time in the ORF23/24 variable region: this region was significantly more stable in extensive farming systems. This result is a first observation suggesting that farm practice may affect the evolutionary dynamics of WSSV. Moreover, these data also suggest that for retrospectively studying the spread of WSSV, it is better to sample from extensive farms than from intensive farms because WSSV populations in extensive farms will be more stable over longer period of time.

Keywords: White spot syndrome virus, shrimp farming, shrimp aquaculture, genetic marker, epidemiology

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INTRODUCTION

White spot syndrome virus (WSSV) has been a scourge on shrimp farming since the early 1990s, causing massive mortality and major damage to many types of shrimp farming operations, from extensive to high-intensity practices (Escobedo-Bonilla *et al.*, 2008). During this period the virus has undergone distinct genotypic change resulting in the occurrence of variants world-wide and in variants with increased fitness and virulence (Marks *et al.*, 2004, 2005). This suggests an adaptive evolution to the novel environment and ecological niche provided by shrimp ponds. In shrimp ponds host density will typically be much higher than in natural habitats. The between- and within-species variation in hosts is also likely to be lower in intensive ponds than in natural environments, and hosts are likely to be stressed due to pond conditions. All of these factors probably contribute to generating a novel environment in which WSSV can thrive and further optimize its fitness.

Shrimp-farming operations are, however, highly varied. Extensive and improved extensive shrimp farms (i) stock shrimp larvae directly from the sea, in part or entirely, (ii) have a relatively low density of shrimp, and (iii) have overlapping generations of shrimp. On the other hand, intensive shrimp farms (i) stock shrimp post-larvae (PL) from hatcheries, and the broodstock can originate from geographic locations far from the farm, (ii) have a relatively high density of shrimp, and (iii) have non-overlapping generations of shrimp. Typically a pond is seeded with postlarvae (PL) and they are subsequently harvested together, after which the pond is drained and cleaned. Because extensive and intensive shrimp farms provide such differing environments, farming practice may lead to differential selection of WSSV genotypes. Moreover, in intensive farming operations, virus populations present in ponds may be largely discontinuous. This discontinuity will arise because (1) regular drainage and cleaning of the pond removes infectious host cadavers or debris, and (2) non-overlapping shrimp generations preclude between-cohort transmission of the virus by infectious shrimp. This may have implications for the genetic composition, evolutionary dynamics and epidemiology of WSSV populations.

Here, we hypothesize that different farming practices will have an effect on WSSV genotype composition and population structure. WSSV isolates were collected from Vietnamese improved extensive and intensive shrimp farms at different geographic locations and different time points. These isolates from each type of farm were genetically characterized, allowing us to test whether farming practice affected genotypic composition and change thereof over time.

METHODS

Classification of shrimp farms

WSSV infected shrimp were purposely collected from shrimp farms with different farming practices. We classified the farms based on farm organization and management, in a manner similar to Nhuong *et al.* (2002) using the following three categories:

(1) Intensive shrimp farming: Pond size varies from 0.2 to 0.6 ha, and a stocking density ranging from 15-30 post larvae per m^2. Shrimp are stocked only once for each crop, industrial shrimp food is used, and water oxygen supply is augmented by machinery. PLs are bought from local hatcheries, but the origin of broodstock is typically unknown. Shrimp crops are harvested after about four months. If there are disease outbreaks, shrimp are quickly harvested and the pond is chemically treated and drained for cleaning prior to new stocking.

(2) Improved extensive farming: Pond size greatly varies, ranging from 1 to 15 ha, including ditches and surrounding dikes. Shrimp seed are trapped from wild stock by making use of tides, and sometimes farmers supplement wild stock with PL from hatcheries (approximately once a month, although this varies greatly between farms). This results in a low stocking density of 1-2 shrimp per m^2. No additional feed is required in this system as shrimp use natural feed in pond. Farmers typically harvest shrimp once or twice a month, based on tides. Large shrimp are trapped by nets as marketable harvest, although small shrimp suffering from disease are also often trapped and hereby removed from the pond.

(3) Shrimp-rice farming: similar to improved extensive farming in terms of farm management, although the plots used are somewhat smaller than improved extensive ponds. In the Mekong Delta of Vietnam, farmers use the same plot to cultivate rice in the rainy season, when freshwater is abundant, and shrimp in the dry season, when saline water is used to flood the plot.

Collection of virus isolates

Shrimp showing disease symptoms characteristic for WSSV were selected, cleaned on the outside with 70% ethanol, and stored in 96% ethanol during transportation to Can Tho University (Vietnam). The ethanol was then removed and samples were stored at -20° C until further processing. All shrimp selected were *Penaeus monodon*. Information on the WSSV infected shrimp collected is recorded in Table 1, and geographic locations are given in Fig. 1.

Genetic characterization of virus isolates

The characteristics of WSSV have been described by Vlak *et al.* (2005) and further International Commission on Taxonomy of Virus (ICTV) updates can be found at http://ictvonline.org. Marks *et al.* (2004) identified five variable regions (see Fig. 2): three loci with variable number of tandem repeats (VNTR; ORF75, ORF94 and ORF125; ORF nomenclature according to Van Hulten *et al.*, 2001), and two loci with large deletions (ORF23/24, ORF14/15). These variable loci have been employed as markers in different studies on different spatiotemporal scales (e.g. Wongteerasupaya *et al.*, 2003; Dieu *et al.,* 2004; Hoa *et al.,* 2005, Marks *et al.*, 2005; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008a, 2008b). Here we employed these five variable regions as markers.

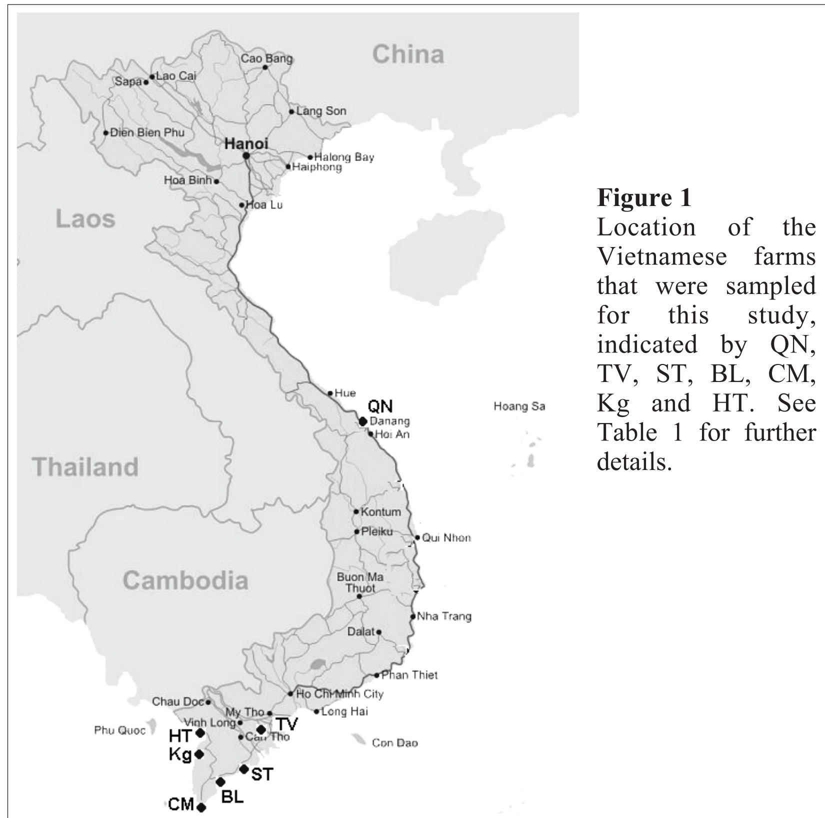

Figure 1
Location of the Vietnamese farms that were sampled for this study, indicated by QN, TV, ST, BL, CM, Kg and HT. See Table 1 for further details.

DNA was extracted from tissue behind the heads of collected shrimp, and screened for the presence of WSSV according to published procedures (Dieu *et al*., 2004). PCR on the genomic variable loci of WSSV was performed with 1µl DNA extract (approximately 250 ng DNA), using Taq DNA polymerase (Promega). Specific PCR primers, conditions used and amplicon lengths are shown in Table 2; PCR for VNTRs is described elsewhere (Dieu

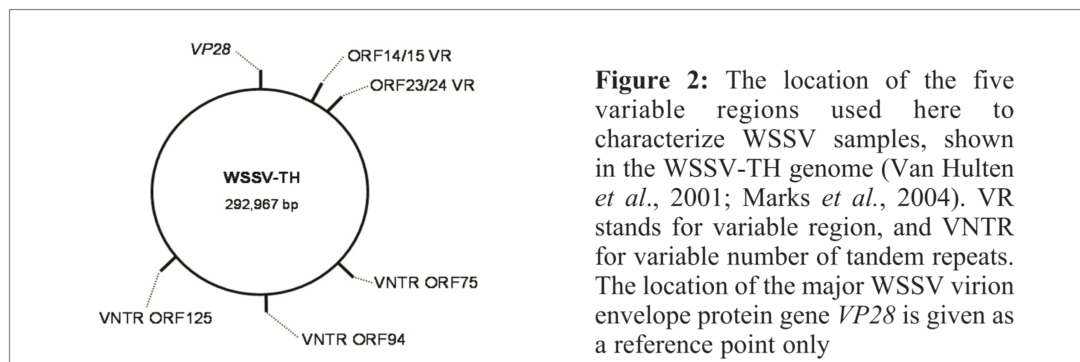

Figure 2: The location of the five variable regions used here to characterize WSSV samples, shown in the WSSV-TH genome (Van Hulten *et al*., 2001; Marks *et al*., 2004). VR stands for variable region, and VNTR for variable number of tandem repeats. The location of the major WSSV virion envelope protein gene *VP28* is given as a reference point only

et al., 2004). PCR products were analyzed, sequenced and computational analysis was done according to published procedures (Dieu *et al*., 2004). For analysis of the deletion loci (ORF14/15 and ORF23/24), two WSSV infected shrimp from each pond were PCR analyzed.

Statistical analysis

To analyze VNTR data we considered the number of repeat units (ORF94 and ORF125). For ORF75, we considered the total length of the repeat region, since this variable locus contains two types of repeats. To analyze data from genomic deletions (ORF14/15 and ORF23/24) we considered the length of the genomic deletion. We refer to these quantitative data as 'locus trait values'. Because the data set is limited, we grouped improved extensive and shrimp-rice farms together as being 'extensive', and compared them to intensive farms.

As a simple test of whether farming practice had an effect on genotypic composition, we performed a Mann-Whitney *U*-test (SPSS 15.0; SPSS Inc., Chicago, IL, USA), with farming practice as the independent variable and locus trait value (RU number, total length of TRs or deletion size) as the dependent variable. We only included data from the years 2002, 2006 and 2008 from the CM site (Ca Mau, Table 1) to avoid biasing our analysis due to high number of samples from a single site and small time intervals (see Table 3). CM 2006 was chosen as the intermediate sample because for that year the VNTR data are complete.

Table 1. WSSV isolates analysed in this study

Region	Province	Place (district)	Farming practice	Origin of post larvae	Date of collection	Abbreviation
Central VN	Quang Nam	Nui Thanh	Intensive	Central region	2003, 2008	QN
South VN	Tra Vinh	Duyen Hai	Intensive	Unknown	2004, 2006	TV
	Ha Tien	Thuan Yen	Intensive	Unknown	2004, 2005	HT
	Kien Giang	Kien Luong	Intensive	Central region	2003, 2005	Kg
	Soc Trang	My Xuyen	Rice-shrimp	Unknown	2002, 2004, 2008	ST
	Bac Lieu	Vinh Loi	Extensive	Unknown	2004, 2008	BL
	Ca Mau	Tan Thanh	Extensive	Local	2002, 2004, 2005, 2006, 2007, 2008	CM

To test whether farming practice had an effect on changes in genotypic composition over time, we first determined whether there was a change in locus trait value between samples from the same site. For sites at which more than two samples were available, we compared the earliest and the latest available samples only. Thus the genotypic data from two samples from different time points represent one event: locus trait values are either the same or

Table 2. Primers used in PCR analysis for the variable loci of WSSV

Primer pair name/ (Detected deletion)	Primer	Sequence (5'-3')	Annealing temp. (°C)/ elongation time (s)	WSSV–CN sequence coordinates	Size (bp) of PCR product for VN samples
VR23/24 –HTvar	Forward	GAGTAGTCTTCAATGGCAATGT	49 / 100	275008-275029	~1200
(10970 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 – CM	Forward	CAGATAATGCAAACACGAGACAC	49 / 75	275794-275816	~500
(11045 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –screen	Forward	CACACTTGAAAAATACACCAG	49 / 75	278179-278199	~550
(8539 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –south	Forward	GTAGTGCATGTTTCTCTAAC	49 / 100	275032-275051	~400
(11866 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –TV	Forward	CTACAACGGCCAAGTCAT	49 / 100	30701-30718*	~1600
(11450 bp)	Reverse	ATGATTGTATTCGTCGAAGG		286706-286687	
VR23/24 –Kg	Forward	CTACAACGGCCAAGTCAT	49 / 100	30701-30718*	~2600
(12166 bp)	Reverse	CGCAATTCTCCTCGCAGTT		32255-32237*	
VR14/15-screen	Forward	GAGATGCGAACCACTAAAAG	49 / 75	22904-22923*	~500/
(6031bp and 5950 bp)	Reverse	ATGGAGGCGAGACTTGC		24157-24141*	600
VR14/15-HT	Forward	GAGATGCGAACCACTAAAAG	49 / 80	22904-22923*	~900
(5138 bp)	Reverse	GAAAAATAAATCACGGGCTAATC		23646-23624*	

* According to WSSV-TH sequence

they are not. We recorded the total number of locus trait value changes for each locus, and then tested whether intensive farms had more changes in locus trait value than extensive farms using a one-sided test of equal proportions (R 2.7.0; The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Genotyping of WSSV isolates

All shrimp samples tested positive for the presence of WSSV using a single step PCR. VNTRs and variable regions were analyzed by PCR for all the studied isolates (Table 3). In order to map deletions in the ORF14/15 and ORF23/24 variable regions, we first performed PCR with the "VR14/15-screen" and "VR23/24-screen" primers on all samples, respectively (Table 2). These primer sets were previously used to detect deletions in six VN-central WSSV isolates (Dieu *et al*., 2004). Those samples that failed to give a PCR product were then analyzed by means of a 'walking PCR' with different primer sets, starting from two ends of variable regions. Genotypes were detected with the corresponding primer set (Table 2). All PCR products were cloned and sequenced to confirm their identity and

Table 3. Genotyping of five variable loci in the WSSV isolates. An asterisk (*) indicates the PCR reaction failed. A double dagger (‡) indicates that the data were excluded from the Mann-Whitney *U*-test (Table 3) to avoid biases. For ORF75 (a) stands for an RU with 102 bp, and (b) for RU with 45 bp.

Farming Practice	Location	Year	ORF75		ORF94	ORF125	ORF14/15	ORF23/24
			RUs (total)	TR Length				
Extensive	ST	2002	abbabb (6)	384	14	8	6031	11,866
		2004	abbab (5)	339	4	5	6031	11,866
		2008	babb (4)	237	*	4	5950	11,866
	BL	2004	babb (4)	237	*	*	6031	11,866
		2008	babb (4)	237	10	7	5950	11,866
	CM	2002	ababb (5)	339	3	4	6031	11,045
		2004‡	*	*	*	*	6031	11,045
		2005‡	abbabb (6)	384	9	*	6031	11,045
		2006	abbab (5)	339	7	5	6031	11,045
		2007‡	ababb (5)	339	*	7	6031	11,045
		2008	babb (4)	237	6	8	5950	11,045
Intensive	QN	2003	ababb (5)	339	10	6	6031	8,539
		2008	abbabb (6)	384	6	7	6031	11,866
	HT	2004	babbb (5)	282	11	6	5138	11,866
		2005	abbab (5)	339	10	5	5950	10,970
	Kg	2003	abbab (5)	339	15	6	6031	12,166
		2005	abbab (5)	339	12	9	6031	11,866
	TV	2004	abbab (5)	384	10	9	6031	11,450
		2006	abab (4)	294	6	7	5950	10,970

map the exact position of the deletion. The location and size of the genomic deletion was determined using WSSV-TH-96-II (acc. no. AY753327; Marks *et al.,* 2005) as a reference sequence for ORF14/15, and WSSV-TW (acc. no. AF440570; Wang *et al.*, 1995; see also Marks *et al.*, 2004) as a reference for ORF23/24.

Effects of farm practice on genotypic composition

A Mann-Whitney *U*-test (Table 4) demonstrated that there was no significant effect of farming practice (extensive or intensive) on any locus trait values (RU number, total length of TRs or deletion size) for all geographic locations.

Effects of farm practice on changes in genotypic composition

A test of equal proportions (Table 5) demonstrated that for the ORF75, ORF94, ORF125 and ORF 14/15 variable regions, there was no effect of farming practice on change in locus trait value in consecutive samples from a single geographic location. However, for the ORF23/24 variable region, there was a significant effect of farming practice on change in locus trait value (i.e. deletion size). For the intensive farms a change in locus trait value for ORF23/24

was always observed (4 changes in 4 observed events). For the extensive farms no change in locus trait value was observed (no changes in 3 observed events included in statistical

Table 4. Comparison of locus trait values between extensive and intensive farms. Note that analysis was performed on different measures (Analysis). A Mann-Whitney *U*-test was performed to test for significant differences in trait value between extensive and intensive farms. No significant *P*-values were found.

Locus	Analysis	Samples	Mean ± SE		Mann-Whitney U-test	
			Extensive	Intensive	Z	P
ORF75	Total length TRs (bp)	16	293.6 ± 22.0	337.5 ± 12.9	-1.441	0.149
ORF94	RU number	14	7.33 ± 1.67	10.00 ± 1.05	-1.377	0.169
ORF125	RU number	15	5.86 ± 0.67	6.88 ± 0.52	-1.173	0.241
ORF14/15	Deletion size (kb)	16	6.00 ± 0.00	5.89 ± 0.11	-0.185	0.854
ORF23/24	Deletion size (kb)	16	11.56 ± 0.15	11.19 ± 0.41	-0.507	0.613

analysis, no changes in 8 observed events in total).

Although we analyzed changes in trait locus values over time, the interval between sampling at different locations was irregular (extensive farms: 5.33 ± 0.58 [mean ± SE]; intensive farms: 2.50 ± 0.87). The mean time interval was twice as long on the extensive farms meaning that our analysis of change in viral genotypes over time is probably conservative.

DISCUSSION

We investigated the effect of extensive or intensive shrimp farming on WSSV genotypic composition and on changes in WSSV genotypic composition over time. We found no effect of shrimp-farming practice on WSSV genotypic composition for any of the five variable loci investigated (Table 4). This suggests that the environments associated with extensive and intensive farms are not divergent enough to impose differential selection for WSSV genotypes. On the other hand, the number of samples was relatively small, making it difficult to draw definitive conclusions from these data alone. More intensive sampling in the future followed by genetic analysis should substantiate this claim.

For four out of five variable loci (ORF75, ORF 94, ORF 125, ORF14/15) we found no

Table 5: Comparison of change in locus trait values over time between extensive and intensive farms. A test of equal proportions was performed to test for significant differences in changes in trait value between extensive and intensive farms. Significant *P*-values are marked with an asterisk (*).

Locus	Changes / Total Observed (Proportion)		Test of equal proportions	
	Extensive	Intensive	X^2	P
ORF75	2 / 3 (0.67)	2 / 4 (0.50)	0.000	0.500
ORF94	1 / 1 (1.00)	4 / 4 (1.00)	-	-
ORF125	2 / 2 (1.00)	4 / 4 (1.00)	-	-
ORF14/15	3 / 3 (1.00)	2 / 4 (0.50)	0.365	0.727
ORF23/24	0 / 3 (0.00)	4 / 4 (1.00)	5.405	0.030*

effect of farming practice on changes in locus trait value over time (RU number, total length of TRs or deletion size; Table 5). For ORF23/24, however, we did find a significant effect of farming practice on change in locus trait value (deletion size); whereas deletion size never changed over time for an extensive farm, it always changed for intensive farms (Tables 3 and 5). Moreover, the average time between sampling was twice as long on the extensive farms as on intensive farms, meaning that viral populations on extensive farms had twice as much time to undergo genetic change. Overall, these data therefore suggest that our hypothesis that WSSV populations on intensive farms will be more variable is correct. An effect of farm practice on viral genotypic stability is probably due to (i) frequent seeding of PLs infected with different virus strains (Withyachumnarnkul, 1999) and (ii) pond drainage and cleaning regimens.

Why do the data suggest an effect only for ORF23/24, and not for the other loci? First, this is a preliminary study with a limited sample size, the power of the statistical test is low. However, there appears to be an interesting trend for the ORF14/15 data. For samples from four out of five geographic locations in which the deletion size changed over time, there was a shift from the 6031 bp deletion to the 5950 bp deletion. Moreover, the 5950 bp deletion was found only in samples from later years (2005-2008; see Table 3). This suggests that there was selection for a genotype carrying this slightly smaller deletion during that period of time. What could have caused the occurrence of selection at most of the sites? In this period of time, *P. vannamei* was widely introduced in Vietnamese shrimp farms, replacing *P. monodon* (Raux *et al.*, 2003; Corsin, 2005). Others have shown that passaging in different host species can result in differential selection of WSSV genotypes (Waikhom *et al.,* 2006), lending credibility to this explanation. Moreover, the 5950 bp deletion appears to be selected for when WSSV samples obtained from *P. monodon* are passaged in *P. vannamei* (B.T.M. Dieu and J.M. Vlak, unpublished data).

We observed no trends in the VNTR data (Tables 2, 3 and 4). The data of Pradeep *et al.* (2008a, 2008b) suggest that VNTR loci (ORF75, ORF94, ORF125) are more variable than deletion loci (ORF14/15, ORF23/24). We have also found a similar trend for the spread of WSSV in Vietnam (Dieu *et al.*, 2004, ibid. 2010). These observations may explain why we did not find an effect of farming practice on locus trait value for VNTRs: variation is generated too rapidly for VNTRs to be useful markers on larger spatial - and temporal - scales.

Our data suggest that extensive farming leads to fewer changes in deletion size for the ORF23/24 variable region, as compared to intensive farming. This result has important ramifications. First, it suggests that virus populations in extensive farms will be more stable than in intensive farms, a result that we hypothesized based on the way ponds are managed under these farming practices. This will have consequences for WSSV evolutionary dynamics. For intensive farming systems, infection of a pond can be an evolutionary dead end as the pond will eventually be drained and cleaned, leading to the destruction of most virions. On the other hand, if ponds are not carefully cleaned and viruses reach into the surroundings by e.g. marine crabs, fresh water prawn *Macrobrachium rosenbergii* (Hossain

et al., 2001) or polychaetes (Vijayan *et al*., 2005), a disease-free intensively-managed pond is then a resource which can be best exploited by highly virulent genotypes, as there is no cost of virulence (i.e. the pond will be drained irrespective of virus behavior). In extensive farming systems the costs of virulence may be maintained i.e. killing the host at any point in time means that host introduced into the pond at a later time cannot be directly infected. This line of thought may also extend to shrimp-rice farming, because the plots used are never completely drained and cleaned. WSSV is known to cause asymptomatic or avirulent infections (Withyachumnarnkul, 1999; Flegel *et al.*, 2004), which may be important for maintaining the virus in low-density host populations, for example by vertical transmission. Genetic composition of WSSV populations may be one factor which determines virulence, as has been shown by Marks *et al.* (2005) and suggested by Hoa *et al.* (2005). There will, however, be many other factors which will determine WSSV virulence, such as farm management (Corsin *et al.*, 2001), temperature (Rahman *et al.*, 2006), salinity (Liu *et al*., 2006), ammonia-N (Jiang *et al*., 2004) and adaptive shrimp responses (Flegel, 2007).

Finally, effects of shrimp farming practice on changes in locus trait value have implications for studying the spread of WSSV by means of molecular epidemiology (Dieu *et al.*, 2004; Pradeep *et al.*, 2008a, 2008b, Dieu *et al.*, 2010). If WSSV populations in intensive farm systems are more variable, this means that the original genotype which colonized the farm - and the surrounding region - is less likely to be maintained in the virus population than in an extensive farm. Hence, if samples are taken retrospectively to determine virus spread (e.g. Dieu *et al.*, 2004, 2010), then it is best to sample from extensive farms, because the virus population sampled is more likely to be representative of the genotypes that were first introduced - or first became predominant - in that area. The effects of farming practice on the stability of WSSV populations should therefore be given consideration in the design of experiments to study the spread and epidemiology of WSSV.

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The utilization of VP28 gene to protect penaeid shrimps from white spot syndrome virus disease: a review

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ABSTRACT

White spot syndrome (WSS) is a viral disease which affects most of the commercially cultivated marine shrimp species all over the world causing significant losses. Deaths occur within days of symptoms and affect a diverse range of commercial crustacean species. A heavy mortality is usually observed in the entire infected population. Survival rates can be anywhere from 30% to zero. Consequently, tools with the capability to effectively and timely detect the diseases have been developed to prevent and contain these pathogens. White spot syndrome virus (WSSV) envelope protein VP28 gene is widely used because its ability to bind to the surface of shrimp epithelial cells and might promote innate immune recognition of WSSV. Its recombinant protein was expressed in various expression systems and used as recombinant vaccine or immunostimulant to increase shrimp survival against WSSV. An anti-body against VP28 was successfully used in virus neutralization assay. A DNA vaccine encoding VP28 gene also provided long-term and high levels of protection against WSSV. Moreover, RNA interference (RNAi) resulted in endogenous RNA sequence-specific degradation to silence VP28 gene expression as well as induced an immune response against virus using non-specific dsRNA in shrimp. However, in order to use VP28 on a commercial scale in the shrimp industry, there are several concerns that need to be addressed such as storage, delivery methodology, protection efficiency; protection period and ensuring that it can be produced in large scale. All these methods might lead to the new strategies to control WSSV using the VP28 gene in future.

Keywords: white spot syndrome virus (WSSV), VP28, penaeid shrimp

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INTRODUCTION

Penaeid shrimp are regarded as the most economically important among the crustacean species. Penaeid shrimp are mainly farmed in Southeast Asia (Thailand, Indonesia, Philippines and Vietnam), Central America (Mexico and Panama) and South America (Ecuador, Peru and Venezuela). High demand for shrimp in the global market leads to the birth of shrimp farming in these developing countries. Shrimp aquaculture started in the 1970s as an industrial activity and developed rapidly with huge increase in number of hatcheries and farms. The demand for shrimps in global market is increasing every year. In 2006, the amount of total shrimp production was over 2.6 million tones and over US$10 billion (www.fao.org/fishery/statistics/global-production/en). However, with the rapid production and development, the shrimp aquaculture industry has encountered serious problems including non-infectious and infectious diseases in every major farming area throughout the world. Identified shrimp pathogens such as bacteria and virus are spreading and new diseases are continuously being identified.

White spot syndrome (WSS) is a viral disease that affects most of the commercially cultivated marine shrimp species all over the world (Flegel, 1997; Lotz, 1997). It was first observed in East Asia in 1992–1993 and has since spread to most shrimp culture countries (Yan *et al*., 2007). WSS manifests itself quickly and deaths occur with days of symptoms. It affects a diverse range of crustacean species (Lo *et al*., 1996; Wang *et al*., 1998) including *Fenneropenaeus chinensis*, *F. indicus*, *F. merguiensis*, *Penaeus monodon, Litopenaeus setiferus, L. stylirostris, and P. vannamei.* Within a week of the infection a heavy mortality begins. Survival rates can be anywhere from 30% to zero, but in many cases the entire pond is wiped out.

WSS is caused by an enveloped double-strand DNA virus called white spot syndrome virus (WSSV), which belong to the family of *Nimaviridae* and the monotypic genus *Whispovirus*. It has an ovoid or ellipsoid to bacilliform shape with a flagella-like appendage at one end of the virion (van Hultan *et al*., 2001a). The genome of three WSSV isolates has been fully sequenced: Thailand 293 kbp (van Hulten *et al*., 2001b); China 305 kbp (Yang *et al*., 2001) and Taiwan 307 kbp (Chen *et al*., 2002). Its genome contain 181 non-overlapping open reading frames, consist of five major proteins named VP28, VP26, VP24, VP19 and VP15 (van Hultan *et al*., 2001b). Two of these major proteins (VP28 and VP19) were characterized as envelope protein (van Hultan *et al*., 2001b). VP28 appears to bind to the surface of shrimp epithelial cells (Yi *et al.,* 2004). Such binding is thought to be mediated by a shrimp VP28-binding protein, PmRab7, suggesting that shrimp possess innate immune recognition of WSSV (Sritunyalucksana *et al*., 2006; Tang *et al*., 2007). Therefore VP28 is wildly used for diagnosis and control WSS although its efficacy varies.

1. Recombinant protein VP28

VP28 recombinant protein (rVP28) has been expressed in various expression systems such as in Gram-negative bacteria, *Escherichia coli*, (Jha *et al*., 2006b; Namikoshi *et al.,* 2004; Witteveldt *et al*., 2004a; Witteveldt *et al*., 2004b; Witteveldt *et al*., 2006), Gram-positive

bacteria (Caipang *et al*., 2008; Mavichak *et al.,* 2009) insect cells (Du *et al*., 2006), yeast (Jha, Xu and Pandey, 2006a; Jha *et al.,* 2007) and silkworms (Xu *et al*., 2006). In this first study, van Hulten *et al.* (2000) expressed the rVP28 in an insect cell. The difference between the actual weight (28 kDa) and the predicted weight (22 kDa) was attributed to the use of an insect expression system that may be the result of post-translational modifications (e.g. glycosylation, phosphorylation). However, using prokaryotic system to express and purify rVP28, it has been demonstrated to protect shrimp against WSSV. Namikoshi *et al.* (2004) demonstrated that *E. coli*-expressed rVP26, rVP28 and inactivated WSSV protected kuruma shrimp (*Marsupenaeus japonicus*). The result showed that injection with rVP28 twice (100µg/1g shrimp/ time) gave highest protection against WSSV with 95% relative percent survival (RPS) (Amend, 1981). Witteveldt *et al.* (2004b) evaluated the potential for vaccination using rVP28 N-terminally fused to maltose binding protein expressed in *E. coli*. The result showed that vaccination with rVP28 (4µg/1g shrimp) gave significantly better survival when challenged two days after vaccination with 33% (RPS), but not after 25 days.

Du *et al.* (2006) have compared the efficacy of rVP28 expressed in *E. coli* and insect cells in crayfish, *Procambarus clarkii*. They found that injecting shrimp with rVP28 expressed in insect cells (2.5µg/1g shrimp) provided greater protection than injection with rVP28 expressed in *E. coli* (2.5µg/1g shrimp). The rVP28 expressed in insect cells gave 95% RPS, while rVP28 expressed in *E. coli* gave 43.5% RPS. They suggested that posttranslational modifications of VP28 may have been correctly performed in insect cells. A similar experiment using prokaryote system expressed rVP28 was conducted by Jha *et al.* (2006a) who investigated the potential of injection vaccine using rVP28 expressed in yeast, *Pichia pastoris*, against WSSV in crayfish, *P. clarkii*. They found that injection of rVP28 2µg/ 1g of crayfish give the protection ability at 3 days post-vaccination at 91% RPS and at 21 days post-vaccination at 78% RPS. Also they investigated the effectiveness of oral, intramuscularly (IM) injection (2µg/ 1g of crayfish) and immersion vaccination against WSSV in crayfish, *P. clarkia* (Jha *et al*., 2006b). The rate of cumulative mortality was higher in case of immersion vaccination. The challenge test on 5th and 21st day post-immersion showed RPS value of 49% each followed by oral vaccination. The challenge test on 3rd and 21st day post-oral administration showed 52% and 49% RPS, respectively and injection vaccination. The challenge test on 3rd and 21st day post-immersion showed 50.5% and 43.1% RPS, respectively. However, there seem to be no significant difference based on the RPS value. Thus, oral administration of rVP28 might be a feasible method to administration because injecting individual shrimp is not practical when dealing with large number of shrimp.

Witteveldt and colleagues expressed VP28 in *E. coli* and demonstrated that, when mixed with commercial shrimp pellets, it significantly enhanced survival against WSS challenge in *P. monodon* (Witteveldt *et al*., 2004a) and *L. vannamei* (Witteveldt *et al*., 2006). The level protection in *L. vannamei* was lower compared to *P. monodon* (RPS values of 50% in *L. vannamei* and 77% in *P. monodon*). Caipang *et al.* (2008) use Gram-positive bacterium, *Brevibacillus brevis*, to produce WSSV rVP28. This Gram-positive bacterium has three advantages: it secretes functional extracellular protein directly into the culture

medium, making it useful for large-scale commercial production and it doesn't produce lipopolysaccharide (LPS) which is normally present in Gram-negative bacteria and is endotoxic to many organisms. They found that feeding with rVP28 25µg/ 1g shrimp for 7 days before challenge with WSSV at 3 days post-feeding showed the highest RPS value at 92.8% RPS. The level of protection in shrimp against the disease significantly decreased at 2 weeks post-feeding and when the dose of feeding was decreased. They suggested that the protection ability of rVP28 might rely on the dose and duration of feeding. Also in our previous study (Mavichak *et al.,* 2009), we used Gram-positive bacteria; *B. choshinensis* expressed N- and C-terminus-lacking and N-terminus-lacking rVP28 to increase recombinant VP28 expression and compared the efficacy of oral administration and intramuscular (IM) injection of rN-VP28. Our finding showed that feeding with N-terminus-lacking recombinant VP28 (5µg/ 1g shrimp/day) for 7 days still protected shrimp against WSSV, while with the same feeding conditions of N-and C-terminus-lacking did not. Moreover, one time IM injection (5µg/ 1g shrimp) of VP28 provided greater protection than oral administration for 7 days (5µg/ 1g shrimp/day). Moreover, we coated rN-VP28 with liposomes to improve rN-VP28 uptake through oral administration. The determination of protection against WSSV showed that feeding with rN-VP28 coated with liposomes (rN-VP28-MLV) for 3 days gave greater protection than feeding with rN-VP28 treatment. However, feeding with rN-VP28-MLV for 7 days gave no significant difference to protection with rN-VP28 treatment (Mavichak *et al*., 2010). In addition, uptake of rN-VP28-MLV and rN-VP28 was compared by histology. In this experiment we coated rN-VP28-MLV and rN-VP28 with fluorescein isothiocyanate (FITC). At 1 h post-administration, a strong green-fluorescent signal was detected in the intestine of shrimp treated with FITC-rN-VP28-MLV but no signal was detected in shrimp treated with FITC-rN-VP28 and rN-VP28. Taken together, our results suggest that the use of liposomes can improve the efficiency of delivering rN-VP28 to shrimp.

Xu *et al.* (2006) determine the efficacy of rVP28 using silkworm expression system in crayfish, *P. clarkii*. The results showed that VP28 treated crayfish gave significantly better survival rate against WSSV than control treatment (94.7% RPS). Jha *et al.* (2007) also evaluated the efficacy of orally administration of rVP28 expressed in yeast, *P. pastoris* against WSSV in *P. clarkii*. They found that the highest RPS value was belonging to the sonicated yeast expressing rVP28 (86% RPS and 72% RPS when challenged with WSSV on 3rd and 21st day post-vaccination). These studies provided the information to protect shrimp using rVP28 against WSSV.

2. Antibody against VP28

Several groups have produced monoclonal and polyclonal antibodies against rVP28 as a tool for diagnosing WSSV (Anil, Shanker and Mohan, 2002; Chaivisuthangkura *et al.,* 2004; Liu *et al.,* 2002; Makesh *et al.,* 2006; Poulos *et al.,* 2001; Shih *et al.,* 2001; Zhan *et al.,* 1999). With the development of hybridoma technology, panels of monoclonal antibodies (MAbs) were produced and used in an indirect immunofluorescence assay (IFA) (Shih *et al.,* 2001; Zhan *et al.,* 1999), immunoperoxidase and whole-mount tissue assays (Poulos *et al.,* 2001) for rapid diagnosis of WSSV. However, tests are not suitable for use by farmers. Furthermore there is evidence that various WSSV geographic isolates differ at the molecular level

(Nadala and Lo, 1998; Lo *et al.,* 1999; Wang, Nunan and Lightner, 2000). Therefore, Anil, Shanker and Mohan. (2002) have produced MAbs to an Indian isolate of WSSV for antigen characterization and to develop a simple and sensitive immunodot test that can be used by farmers. Liu *et al.* (2002) produced MAbs specific to VP28 and developed a MAb-based antigen-capture enzyme-linked immunosorbent assay (Ac-ELISA) for detection of WSSV antigen from shrimp tissues and hemolymph. Makesh *et al.* (2006) developed MAbs that reacted with VP28, to diagnose WSSV infection in *P. monodon.* The test had an analytical sensitivity of 625pg of purified virus.

Moreover, antibodies raised against individual viral envelope proteins have been successfully used in neutralization assays to identify proteins involved in virus entry during infection (van Hulten *et al.,* 2001a; van Hulten *et al.,* 2001b: Wu, Wang and Zhang, 2005), as well as to prevent WSSV infection (Kim *et al.,* 2004; Musthaq *et al.,* 2006; Natividad *et al.,* 2007; Robalino *et al.,* 2006;). Kim *et al.* (2004) reported that egg yolk antibodies (IgY) against truncated VP28 fused with truncated VP19 WSSV enveloped proteins, TrVP28:19, were able to neutralize WSSV in challenge trials with *F. chinensis*. Robalino *et al.* (2006) used polyclonal and monoclonal antibodies against VP28 from rabbit serum to inactivate WSSV in *L. vannamei.* Mustaq *et al.* (2006) used various VP28 polyclonal antiserum concentrations to neutralize WSSV in *P. monodon.* The results indicate that WSSV can be neutralized by VP28 antiserum in a dose-dependent manner. Similarly, Natividad *et al.* (2007) demonstrated that a MAb against VP28 was effective in protection of *M. japonicus* from WSSV. These results indicate that antibody against VP28 will be useful for developing new strategies to control WSSV infection.

3. DNA vaccine

DNA-based vaccination constitutes one of the most recent approaches to vaccine development. Vaccination of plasmids carrying genes under the control of the CMV promoter was found to induce protective immunity to many pathogen diseases in farmed animals (Van Drunen *et al.,* 2000). Therefore, the potential of DNA vaccination strategy in shrimp using VP28 gene against WSSV was investigated. Rout *et al*. (2007) constructed DNA vaccines using vector pVAX1 (Invitrogen, Carlsbad, CA, USA), which containing CMV promoter. Each vector carried a WSSV gene (VP15, VP28, VP35 and VP281). The vaccines were injected IM to *P. monodon*. PCR was used to detect the plasmid carrying VP28 gene distribution in shrimp tissues (hemolymph, pleopods, telson, gills, hepatopancreas and gut). They found that on days 2-15 post vaccination, VP28 was detected in all tissues. However, on day 45 it was not detected in hemolymph and day 60 it was not detected in hemolymph and hepatopancreas. Rout *et al*. (2007) also compared the effects of rVP28 (2 IM doses of 1µg/ 1g of shrimp on days 1 and 5) and a DNA vaccine (single dose of 2µg/ 1g of shrimp). On day 14, RPS was better for the rVP28 group (65%) than for the DNA vaccine group (48%). However, the DNA vaccine provided longer protection (30% RPS on day 60 vs. -11.6% for the rVP28 group). These results suggested that a DNA vaccine could provide long-term antigen expression through a genetic immunization strategy. Therefore, it offered continuing protection ability against WSSV at least up to 60 days.

Similar results were obtained by Rajesh Kumar and co-workers (2008) using a DNA vaccine constructed from the pcDNA 3.1 expression vector, which also has the CMV promoter and the VP28 gene. The VP28 was detected by PCR in abdominal muscle, gill, hepatopancreas, pleopods and gut on days 3, 15 and 30 post-immunization. The DNA vaccine protected against WSSV up to 30 days post-immunization (RPS values were 90% on day 7 and 57% on day 30). Prophenoloxidase (proPO) and superoxide dismutase (SOD) activities were measured to assess the immunological response to the DNA vaccine encoding VP28. Both activities increased significantly on day 7 post-immunization and then gradually decreased. Both activities were higher than those in the control groups till day 30. However, the superoxide anion concentration did not change significantly during the experiment.

Attenuated *Salmonella typhimurium* have been used as a system for expressing foreign antigens under the control of a eukaryotic promoter for vertebrate host cells (Abudul Wahid and Faubert, 2007; Chen *et al*., 2006; Du and Wang, 2005; Li *et al.,* 2006). Ning *et al.* (2009) used attenuated *S. typhimurium* to express a DNA vaccine encoding VP28 and orally administered them to crayfish, *Procambarus clarkii*. The DNA vaccine was detected in hemolymph, ovary, male reproduction system, heart, hepatopancreas and abdominal muscle from day 4 to day 30 post-immunization. By the end of the experiment (day 30 post-immunization), the vaccine was detected by reverse transcription PCR only in the abdominal muscle. These results correlated with cumulative mortality data following WSSV challenge. The RPS values following WSSV challenge were 83% on day 7, 67% on day 15 and 57% on day 25 post-vaccination. Together, these findings suggest that VP28 DNA vaccine can be easily produced and can provide long-term protection against WSSV.

4. RNA interference

RNA interference (RNAi) is one of the new procedures to control WSS by induces sequence-specific antiviral silencing. Moreover, nonspecific double stranded RNA (dsRNA) was reported to induce shrimp innate immunity (Robalino *et al.,* 2004). Using VP28 dsRNA sequence-specific silencing was successfully demonstrated by Robalino *et al.* (2006). They found that injection with VP28 dsRNA (5µg/ 1g of *L. vannamei*) could increase shrimp survival rate against WSSV, while, sequence-nonspecific dsRNA caused only a delay in the onset of mortality. Westenberg *et al.* (2005) hypothesized that delivery of short interference RNA (siRNA), rather than large dsRNA provided a specific anti-viral response. Therefore, they injected siRNA against VP28 (1.6Mol/1g of *P. monodon*) and challenge with WSSV. The results showed that significantly lower mortality rate was observed in shrimp treated with VP28 siRNA but not significantly different from shrimp treated with sequence-nonspecific siRNA (GFP). Xu and co-workers (Xu, Han and Zhang, 2007) demonstrated the potential for the gene function research and therapeutic treatment of WSSV using four different VP28 siRNAs. However, only injected with VP28 siRNA (3.2g/ 1g of *M. japonicus)* consistent with the design rule for RNAi (Elbashir *et al.,* 2002) could increase the shrimp survival against WSSV. In order to produce high amount of dsRNA, *E. coli* HT115(DE3) the expression of VP28 dsRNA was demonstrated by Sarathi and colleagues (Sarathi *et al.,* 2008a; Sarathi *et al.,* 2008b). VP28 dsRNA was injected in to *P. monodon* (2.5g/ 1 g shrimp) (Sarathi *et al.,* 2008a). RT-PCR analysis was employed to confirm gene silencing effected

after challenge with WSSV. Shrimp treated with VP28 dsRNA did not present VP28 gene at the transcription level. VP28 dsRNA significantly increased shrimp survival rate against WSSV. In addition, they orally administed VP28 dsRNA using bacterially expressed VP28 dsRNA (Sarathi *et al.,* 2008b). The inactivated *E. coli* expressed VP28 dsRNA was mixed with the shrimp commercial pellets and fed to shrimp. Also the purified dsRNA from VP28 dsRNA expressed *E. coli* was coated with chitosan and mixed with shrimp commercial pellet before feeding to shrimp followed by challenge with WSSV. PCR and Western blot analysis were employed to confirm the gene silencing effected after challenged with WSSV. The RT-PCR and Western blot analysis result showed that inactivated *E. coli* expressed VP28 dsRNA and chitosan coated purified VP28 dsRNA treatments were not present VP28 after challenged with WSSV. However, inactivated *E. coli* expressed VP28 dsRNA and chitosan coated purified VP28 dsRNA treatments gave 32% and 63% mortality, respectively, while all control treatments gave 100% mortality after challenge with WSSV. All finding suggested that RNAi showed potential to increase shrimp survival rate against WSSV.

CONCLUSION AND DISCUSSION

Although PCR, which is used to screen post larvae for WSSV, is highly sensitive in WSSV detection, there are practical limitations to its widespread application, including the high cost involved in the setting up laboratory with special equipment and well-trained personnel (Sithigorngul *et al.,* 2000). Furthermore, Lo *et al.* (1998) have indicated that results positive by the highly sensitive 2-step PCR (nested PCR) are not always associated with pond out-breaks and may have limited value for field prognosis. However, 1-step PCR is strongly associated (Withyachumnarnkul, 1999). Some immunological protocols using polyclonal antibodies to whole virus antigens have also been reported (Nadala *et al.,* 1997) but their efficiency is affected by non-specific and false-positive reactions. Therefore, the use of rVP28 to produce monoclonal and polyclonal antibodies targeting rVP28 for diagnosis was suggested (Anil, Shanker and Mohan*,* 2002; Chaivisuthangkura *et al.,* 2004; Liu *et al.,* 2002; Makesh *et al.,* 2006; Poulos *et al.,* 2001; Shih *et al.,* 2001; Zhan *et al.,* 1999). *In vivo* neutralization experiments with neutralizing antibodies have been used for many viruses and have led to passive immunization strategies for WSSV (Kim *et al.,* 2004; Musthaq *et al.,* 2006; Natividad *et al.,* 2007; Robalino *et al.,* 2006; van Hulten *et al*., 2001b; Wu, Wang and Zhang, 2005).

Recombinant VP28 is wildly used to control WSS although the protection mechanism is unclear and its efficacy varies. One possible mechanism of rVP28 is that it binds to shrimp VP28 binding protein, preventing the binding of WSSV to cells (Xu *et al.,* 2006). Similarly, mixing anti-PmRab7 antibody with WSSV before it was inject to shrimp was found to increase survival rate against WSS (Srituyalucksana *et al.,* 2006) and by using dsRNA to suppress expression of PmRab7 (Ongvarrapone *et al.,* 2008). Another possibility suggested by many studies is that rVP28 stimulates shrimp immunity or acts as a vaccine (Bright Singh *et al.,* 2005; Caipang *et al.*, 2008; Chang *et al.,* 2003; Du *et al.,* 2006; Jha *et al.,* 2006a; Jha *et al.,* 2006b; Jha *et al*., 2007; Mavichak *et al*., 2009; Rout *et al.*, 2007; Song *et al.,* 1997; Takahashi *et al.,* 2000; Vaseeharan *et al.,* 2006; Witteveldt *et al.,* 2004a;

Witteveldt *et al.*, 2004b; Witteveldt *et al.,* 2006). Although, the evidence that rVP28 increases the shrimp survival rate against WSSV, the protection ability of rVP28 varies and seems to rely on the amount of proteins received (Caipang *et al.,* 2008; Jha *et al*., 2006b). However, most traditional rVP28 are not cost-effective because they cannot be stored at room temperature. Therefore, there is a need to improve the "storage" of protein-based vaccine to make it more convenient and effective. The alternative expression methods such as plant-based systems that would address "storage" problems should be pursued in future studies.

DNA vaccine is prepared using expression plasmid containing the protective gene for the antigen. It confers high and long-term protection against WSSV. Moreover, it can be stored at ambient temperature; it is easier than recombinant protein vaccine to be prepared at large scales and orally administrated which is practical for shrimp farming. For these reasons, DNA vaccine seems to be the best answer to control WSSV.

The sequence-specific effects of double-stranded RNA (dsRNA) that result in endogenous RNA degradation are widely conserved and probably present in most invertebrates. In contrast, inducing immune response against virus using non-specific dsRNA was demonstrated in shrimp (Robalino *et al.,* 2004). Therefore, dsRNA can engage both innate immune pathways and an RNAi-like mechanism to stimulate potent antiviral immunity in shrimp. From the previous reported, VP28 dsRNA showed the potential to prevent shrimp from WSSV and could be applied for commercial scale because it could produce in large scale using bacterially expressed dsRNA system (Sarathi *et al.,* 2008a; Sarathi *et al.,* 2008b). Moreover, the oral administration strategy is possible, although its efficacy is lower than IM injection method. Therefore, the delivery method needs to be studied using adjuvant or carrier to improve the efficacy of dsRNA oral administration.

In conclusion, the potential of using VP28 gene to diagnose and control WSSV has been studied recently. However, in order to use VP28 in commercial scale in the shrimp industry, there are several issues that need to be addressed including prolonged storage, improving delivery method, increasing its efficiency, the continues long-term protection and ensuring that it can be produced in large scale. Therefore, in the future studies, these issues and also new strategies to control WSSV should be studied to apply to shrimp commercial farming.

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Prophenoloxidase has a role in innate immunity in penaeid shrimp

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ABSTRACT

Melanization, which is brought about by the activation of the prophenoloxidase (proPO) cascade, plays an essential role in the invertebrate immune response. It is triggered by minute amounts of microbial cell wall components leading to the activation of the enzyme proPO, which converts phenols into quinones and eventually resulting to the formation of melanin. Recent studies however, revealed that the importance of proPO in invertebrate immune response, in particular towards microbial pathogens, seems to be varied. Here, we discuss the function of proPO in penaeid shrimp, a commercially important aquaculture species, and its importance to shrimp survival.

Keywords: shrimp, prophenoloxidase (proPO), melanization

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INTRODUCTION

A major and growing problem facing shrimp aquaculture is disease, which is largely due to the intensification of shrimp farming systems in response to the surging demand. Diseases caused by pathogenic organisms are responsible for heavy production losses in shrimp farms worldwide (Bondad-Reantaso *et al*., 2005). Most notable examples are *Vibrio* species and bacilliform viruses, such as the White Spot Syndrome Virus (WSSV), which were shown to cause mass mortalities within days after infection and can rapidly spread resulting in low survival rates (Lightner, 1996; Saulnier *et al.*, 2000). There is, therefore, a need for a better understanding of the shrimp immune system in order to develop measures to lessen the effects of these diseases and hence to ensure the long term viability of the shrimp industry.

Although shrimp are devoid of an adaptive immune system, they possess an innate immune system that effectively protects them from harmful microorganisms (Lee and Söderhäll, 2002). This includes a rigid exoskeleton, the elimination of microbes through encapsulation, nodule formation or phagocytosis, release of antimicrobial peptides, clot formation, and melanization through the activation of the prophenoloxidase (proPO) cascade. The proPO cascade constitutes a major component of the shrimp humoral response and is triggered by very low amounts of bacterial cell wall components such as peptidoglycans, lipopolysaccharides and β-glucans (Hernández-López *et al*., 1996; Söderhäll and Cerenius, 1998). Here, we discuss the role of proPO in the shrimp immune response and its importance to the survival of penaeid shrimp.

ProPO activation

The proPO cascade in shrimp is set off in a stepwise process with the recognition of bacterial cell wall components by pattern recognition proteins. This process in turn, initiates the activation of a serine protease cascade that leads to the conversion of the proPO-activating enzyme (PPAE) to an active proteinase that converts the inactive enzyme precursor, proPO, into phenoloxidase (PO). PO, a copper containing oxidase, eventually catalyzes the oxidation of tyrosine to produce toxic quinone substances and other short-lived reaction intermediates that lead to the formation of melanin. It has been shown that melanin binds to the surface of bacteria and increase the adhesion of haemocytes to bacteria, thus accelerating their removal by nodule formation (Cerenius *et al.*, 2008; da Silva, 2002). In shrimp, proPO has been shown to be localized in the haemocytes (Ai *et al.*, 2009; Lai *et al.*, 2005). In the last decade, many studies have investigated the various aspects of proPO in different shrimp species (Table 1).

ProPO function in shrimp

In invertebrates, the importance of proPO differs, particularly on their survival. In fruit flies, *Drosophila melanogaster*, proPO activation increases the effectiveness of other immune reactions (Tang *et al.*, 2006) and mutant strains that are incapable of melanization tend to be more susceptible to infections (Braun *et al.*, 1998; Lemaitre *et al.*, 1995). In contrast, a separate study, using the same species, showed that proPO activation is not required against microbial infection (Leclerc *et al.*, 2006). On the other hand in mosquitoes, proPO

and melanization is altogether unimportant and is not required for their survival against some bacteria and microbes (Michel *et al.*, 2006; Schnitger *et al.*, 2007). In crayfish, a close relative of shrimp, proPO was reported to be essential for defense against *Aeromonas hydrophila* infections (Liu *et al.*, 2007).

Table 1
Prophenoloxidase (proPO) studies in shrimp

Species	Study	Author/s (Year)
Marsupenaeus japonicus	ProPO cloning, characterization	(Adachi *et al.*, 1999)
	Gene silencing	(Fagutao *et al*., 2009)
Penaeus monodon	Cloning and gene silencing	(Amparyup *et al*., 2009)
	Cloning	(Sritunyalucksana *et al*., 1999)
Penaeus semisculcatus	Cloning and sequencing	unpublished
Penaeus vannamei	ProPO cloning, characterization	(Lai *et al.*, 2005)
	Effect of temperature on proPO	(Pan *et al*., 2008)
	Tissue distribution of proPO transcript	(Wang *et al*., 2006)
	ProPO characterization after Vibrio alginolyticus infection	(Yeh *et al*., 2009)
	Effect of lipopolysaccharides on proPO expression	(Okumura, 2007)
Macrobrachium rosenbergii	Cloning, characterization	(Liu *et al.*, 2006)
	Cloning	(Lu *et al.*., 2006)
Penaeus californiensis	Activation of the proPO cascade	(Hernández-López *et al*., 1996)
	Cloning	(Gollas-Galvan *et al*., 1999)
	Effect of Calcium on proPO	(Gollas-Galván *et al*., 1997)
Fenneropenaeus chinensis	ProPO cloning, characterization	(Gao *et al*., 2009)

In shrimp, proPO was found to be required for defense against microbial pathogens. In Pacific white shrimp, *Litopenaeus vannamei*, proPO was shown to be involved in acute-phase immune defense against *Vibrio alginolyticus* and was also found to be regulated by ecdysone, a hormone that promotes growth and controls molting, which suggests that it may participate in other physiological processes (Yeh *et al.*, 2009). Meanwhile, the expression

of two different forms of proPO in *L. vannamei* was found to be inhibited by WSSV infection (Ai *et al.*, 2008; Ai *et al.*, 2009). In Chinese shrimp, *Fenneropenaeus chinensis*, proPO expression increased after challenge with *V. anguillarum* (Gao *et al.*, 2009). In black tiger shrimp, *Penaeus monodon*, gene silencing of proPO and its activating enzyme PPAE, resulted in a substantial reduction in total PO activity and increased susceptibility to *V. harveyi* infections (Amparyup *et al.*, 2009; Charoensapsri *et al*., 2009). Gene silencing of proPO in kuruma shrimp, *Marsupenaeus japonicus*, resulted in increased bacterial counts in the haemolymph and increased mortality even in the absence of bacterial challenge (Fagutao *et al.*, 2009). ProPO-depleted shrimp were also found to have lower haemocyte counts than control samples and to have significantly down-regulated expressions of antimicrobial peptides such as lysozyme, crustin and penaedin (Fagutao *et al*., 2009).

It is therefore apparent that, unlike flies and mosquitoes, proPO in shrimp plays an essential role in the innate immune response particularly against microbial pathogens and is important for shrimp survival. However, it is unclear whether proPO is involved in other physiological processes. Studies on how to increase or stimulate proPO expression in shrimp for application to actual farm conditions may help to improve the chances of survival during disease outbreaks.

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Hemocyte marker proteins and regulation of the proPO system in a crustacean, *Pacifastacus leniusculus*

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ABSTRACT

In crustaceans and other invertebrates, hemocytes are essential in immunity. The development of new hemocytes is important in the defence mechanisms in invertebrates. In crustaceans, new hemocytes which are synthesized in a specific organ called the hematopoietic tissue (Hpt). The hemocytes are produced and partly differentiated in the Hpt before they are released into the hemolymph and become freely circulating peripheral hemocytes. The final step of differentiation to mature hemocytes containing prophenoloxidase (proPO) takes place in the hemolymph.

This paper presents the studies to identify proteins associated with development of different hemocyte types and a negative regulator of phenoloxidase-induced melanization in the freshwater crayfish *Pacifastacus leniusculus.*

To obtain tools for more detailed investigations about the connection between semigranular cells, granular cells and precursor cells in Hpt of freshwater crayfish, *Pacifastacus leniusculus* and possibly also in other crustaceans, we have used two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS) to identify specific proteins expressed in different hemocyte types differentially. In this study we report the specific expression of some genes in different hemocyte lineages and their transcript levels in Hpt cells in normal or previously laminarin or LPS treated animals analyzed by RT-PCR. Moreover, RNA inference experiments were also included to study the differentiation of Hpt cells.

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Melanin formation is essential for host defence in crustaceans and insects, and this process needs to be tightly regulated since unwanted production of quinone intermediates or melanization is also dangerous to the animal when they are not synthesized appropriately. We also report the presence in the crayfish *Pacifastacus leniusculus* of a plasma protein with a distinctly similar function as mealworm *Tenebrio molitor* melanization inhibiting protein (MIP). Crayfish MIP as well as *Tenebrio* MIP interfere with the melanization reaction from quinine compounds to melanin, but do not affect phenoloxidase activity by themselves instead. Interestingly, this protein has a completely different structure from *Tenebrio* MIP and is similar to vertebrate ficolin and horseshoe crab Tachylectin 5. Moreover, an Asp-rich region similar to that found in ficolins that is likely to be involved in Ca^{2+}-binding is present in crayfish MIP. However, crayfish MIP did not show any hemagglutinating activity which is common for the vertebrate ficolins. The crayfish MIP is very efficient in inhibiting activation of the proPO-system and thus functions as an important regulatory molecule to prevent unwanted proPO activation in the crayfish.

Key words: *Pacifastacus leniusculus*, hemocyte marker protein, proPO system

INTRODUCTION

Background

Crustacean aquaculture production is an important income for many developing or low-income countries (Rosenberry, 1998). The increase in aquaculture also has many negative effects, for instance, it causes environmental destruction and the farming often faces severe disease problems. To optimize aquaculture condition and avoid disease outbreaks, the development of tools for rapid recognition and control of pathogens are imminently needed (Bachère, 2000).

Crayfish are immunologically related to other more economically important crustaceans, so insight into crayfish immunity and their defence system is valuable for aquaculture development and good for optimizing farming conditions.

Vertebrate immunity is composed of innate and adaptive response, but invertebrates, including arthropods, only rely on very sensitive innate immune system since they lack true antibodies and adaptive immunity. The innate immune system seems to be enough to protect them against infections or intruders, and it includes cellular and humoral mechanisms, both of which are activated upon immune challenge. The cellular response mediated by hemocytes in hemolymph involves nodule formation, phagocytosis, encapsularion of pathogens and coagulation (Ratcliff *et al.,* 1985, Johansson and Söderhäll, 1989; Theopold *et al*., 2002;). Moreover, hemocytes are also involved in another cellular response, melanization, which is activated immediately upon injury/infection and normally is localized to the place of injury or the surfaces of invading microorganisms, so that toxic phenol intermediates or melanin

are released and the intruders are immobilized and killed Söderhäll and Cerenius, 1998). Humoral defense is characterized by synthesis and secretion of immune components after challenge, for example: antimicrobial peptides (AMPs) can accumulate in hemolymph to defend against invading microorganisms (Lemaitre and Hoffmann, 2007). But it is important to keep in mind that cellular and humoral responses are connected to each other, and cannot be separated completely in the immune response.

There are various strategies for the invertebrates to combat different invading pathogens, but most of them are evolutionarily conserved such as: activation of phagocytic cells, production of AMPs and generation of toxic or reactive oxygen species (ROS).

In decades, progress has been made in different aspects of crustacean immune system, especially the understanding at molecular and biochemical level of some highly conserved immune response pathways (eg. Melanization, production of AMPs and clotting) (Bachère *et al*., 2004; Cerenius and Söderhäll 2004, Sritunyalucksana and Söderhäll, 2000). However, the research of these animals is being hampered by a lack of access to knowledge of genetic data and of well-developed cell lines. Therefore, the hematopoietic tissue (Hpt) cell cultures technique developed in the freshwater crayfish *Pacifastacus leniusculus*, provide a useful tool for gene functional studies in crustaceans (Söderhäll *et al*., 2005), since an efficient method for RNA silencing is at hand for these cells (Liu and Söderhäll, 2007).

Hematopoiesis

Hematopoiesis is the formation and development of new hemocytes and/or blood cells. This is the process whereby undifferentiated hematopoietic stem cells develop into mature cells involving proliferation, commitment and differentiation (Barreda and Belosevic 2001; Medvinsky and Dzierzak 1999). In crustaceans' three morphologically different classes of hemocytes, hyaline, semigranular cells (SGCs), and granular cells (GCs) are observed within the hemolymph, and all of them are important in immobilizing or destroying invasive pathogens (Johansson *et al*., 2000; Cerenius *et al*., 2008). Hyaline and SGCs are involved in phagocytosis (Söderhäll, 1986), whereas the GCs are responsible for storage and release of the proPO system and also antimicrobial peptides (Sricharoen *et al*., 2005). SGCs and GCs can be cytotoxic and lyse foreign eukaryotic cells (Söderhäll, 1985). In crustaceans, the number of free hemocytes can vary a lot in different individuals and also vary in response to environmental stress, hypoxia, endocrine activity during the moulting cycle and infection (Jiravanichpaisal *et al.,* 2006; Johansson *et al.,* 2000; Le Moullac *et al*., 1998; Söderhäll *et al*., 2003). In addition, the hemocytes number drops dramatically or nearly disappears when the crayfish is unhealthy, dying or infected with virus such as white spot syndrome virus (WSSV) (personal observation). Generally, hemocytes do not divide in the circulatory system of most crustaceans (Söderhäll and Cerenius, 1992; Jiravanichpaisal *et al*., 2006). Thus, new hemocytes need to be continuously and proportionally produced from a separate organ called as hematopoietic tissue. However, there are not many studies published elucidating the mechanisms by which blood cells are released into the circulation in crustaceans. In the crayfish *P. leniusculus*, mature hemocytes come from the sheet-like Hpt which is surrounded by connective tissue and situated on dorsal sides of the stomach (Chaga *et al*., 1995).

In *P. leniusculus* the Hpt cells were studied by light and electron microscopy and the cells were subdivided into five morphologically different cell types that might correspond to different developmental stages of SGCs and GCs (Chaga *et al*., 1995; Johansson *et al*., 2000). The connection between the circulating hemocytes and the hemocyte precursors in the Hpt is still unclear, and the proposed hemocyte lineages have mainly been based on morphological characters. For instance, morphological studies of the hematopoietic tissue have been carried out in blue crab *Carcinus sapidus* (Johnson 1987), shrimp *Sicyonia ingentis* (Hose *et al*., 1992), the lobster *Homarus americanus* (Martin *et al*., 1993), and the black tiger shrimp *P. monodon* (van de Braak *et al*., 2002), but these studies did not reveal any details about maturation and release of the hemocyte in these crustaceans. Data also did not show that the new synthesized hemocytes were released directly from the Hpt or similar tissue, or stored somewhere and released upon activation when they were needed.

Hematopoiesis is the lifelong production of blood cells and is tightly regulated by the various transcription factors that promote or limit cell diversification (Orkin, 1998; Orkin 2000; Sieweke and Graf, 1998). Several hematopoietic transcription factors have been characterized and those are conserved across taxonomic groups including both protostomian and deuterostomian organisms, ranging from flies to humans (Fossett *et al*., 2001b). The crystal cells and the plasmatocytes are two primary blood cell lineages in *D. melanogaster* embryo, and they develop from a common hemocyte progenitor expressing GATA protein Serpent (Srp) (Lebestky *et al*., 2000). The other genes encoding transcriptions factors are: glial cell missing (Gcm), U-shaped (Ush), Lozenge (Lz) also have been identified to be involved in the hematopoietic lineage commitment in *D. melanogaster*. Gcm promotes plasmatocyte development; Ush limits crystal cell development, while Lz promotes crystal cell development; Srp acts upstream of the other factors and is required for late plasmatocyte differentiation. (Fossett and Schulz, 2001a; Lebestky *et al*., 2000; Rehorn *et al*., 1996). In *P. leniusculus*, also some transcription factor proteins have been found. The Hpt cells were shown to be actively proliferating and the Lz-homologue *Pl*Runt was shown to be important in crayfish hematopoiesis (Söderhäll *et al*., 2003). The number of hemocytes dramatically decreased when the crayfishes were injected with a β-1, 3-glucan, resulting in an accelerated maturation of hemocyte precursors in the Hpt followed by release into the circulation of new hemocytes that later develop into functional SGCs and GCs expressing the proPO transcript (Söderhäll *et al*., 2003). In crayfish *P. leniusculus*, all mature hemocytes are expressing proPO while less than 3% of the Hpt cells were found to express this transcript, so we suggested the presence of the proPO transcript can be used as a putative marker for final hemocyte maturation (Söderhäll *et al*., 2003). Moreover, an endogenous cytokine named as astakine from crayfish *P. leniusculus*, containing a prokineticin domain and is critical in the differentiation and growth of hematopoietic stem cells *in vitro* and *in vivo* (Söderhäll *et al*., 2005; Lin *et al*., 2008) (Fig. 1).

In the five different morphological type cells as suggested by Chaga *et al.* (1995), proPO mRNA is restricted to type 4 cells, and expression of *Pl*Runt in Hpt in this stage is low in all cell types. A short period prior to the release of the cells into circulation, the *Pl*Runt transcript was induced significantly whereas proPO expression was delayed until

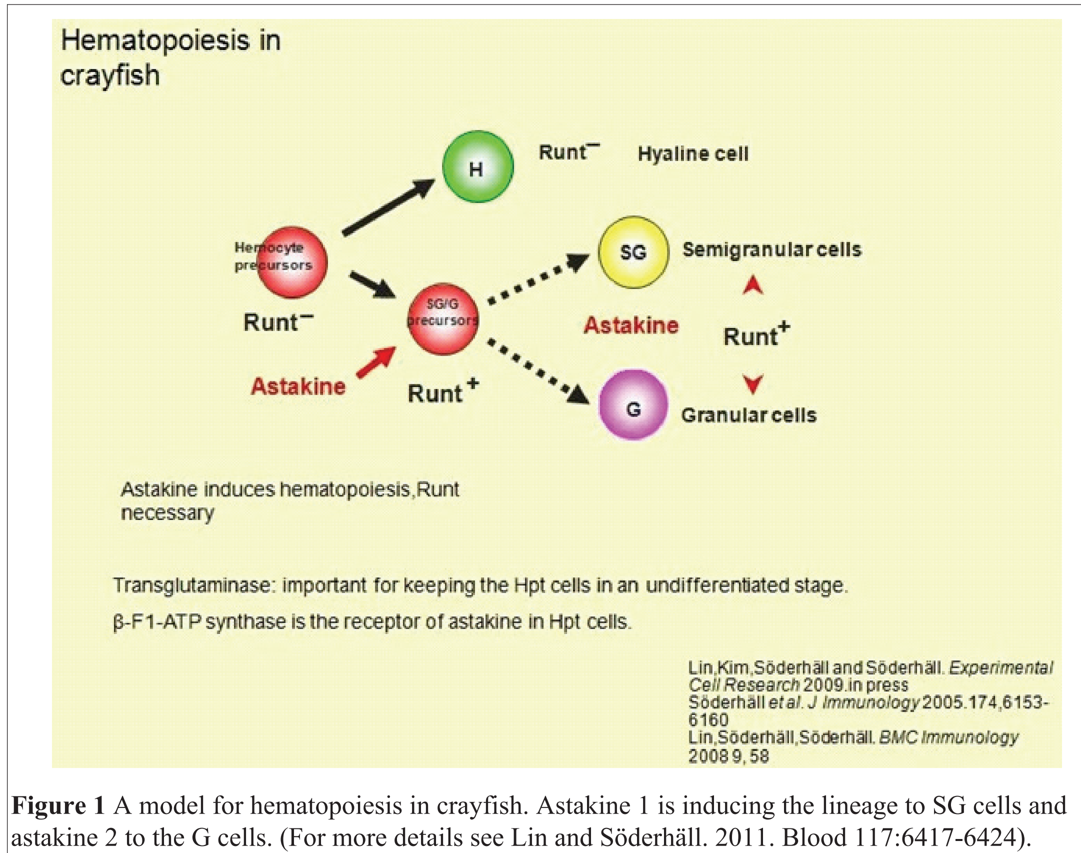

Figure 1 A model for hematopoiesis in crayfish. Astakine 1 is inducing the lineage to SG cells and astakine 2 to the G cells. (For more details see Lin and Söderhäll. 2011. Blood 117:6417-6424).

the hemocytes matured and reached the circulation (Söderhäll *et al*., 2003), showing that the final stage of development into functional semigranular or granular hemocytes happen after their release from the hematopoietic tissue. Morphological observation also showed the presence of these newly released hemocytes with an appearance more similar to type 4 cells (Söderhäll *et al*., 2003).

Absence of proPO transcript in the majority of Hpt cells indicates that this protein is not required until the cells are released into the circulation, which is of considerable interest since this protein is the very important protein in innate immune reactions as well as during sclerotization of the cuticle (Söderhäll *et al*., 2003; Söderhäll and Cerenius, 1998).

In crustaceans and other invertebrates the circulating hemocytes play the most important role in the protection of the animal against invading microorganisms. Therefore, hematopoiesis is essential to produce new hemocytes which can particpate in the cellaular defence. Recently we have shown that crayfish hemocytes have different marker proteins which can be used to understand the differentiation of hemocytes from Hpt cells. Thus we found that proliferating cell nuclear antigen (PCNA) is specific for Hpt cells, a Kazal

proteinase inhibitor for SG cells and Superoxid dismutase for G cells (Wu et. al. 2008) and also that transglutaminase is essential for the maintenance of the Hpt cells in their stem cell form. Further we recently also isolated and characterised the membrane receptor for the cytokine, astakine which is invoved in hematopoiesis (Lin *et al*., 2007).

Prophenoloxidase activating system (proPO-system) and melanization

The melanization reaction is an important immune reaction in arthropods (Cerenius *et al*., 2008) (Figure 2). It is a rapid immune response important in encapsulation and killing of microbial pathogens in invertebrates and melanin depositions are often observed on the surface of invading parasites in the hemocoel or at the site of cuticular injury. Melanin synthesis is achieved by the proPO activation system, a proteolytic cascade similar to vertebrate complement (Cerenius *et al*., 2008). In invertebrates, the proPO cascade (Figure 3) is a very efficient non-self-recognition system, which can be triggered by minute amount of microbial components, e.g. β-1, 3-glucans from fungi and lipopolysaccharides (LPSs)/ peptidoglycans (PGNs) from bacteria, and includes several immune defense proteins resulting in melanization, cell adhesion encapsulation, and phagocytosis. In crustaceans, the proPO-system including several pattern recognition proteins (PRPs), such as β-1, 3-glucans binding protein (βGBP) and lipopolysaccharide and β-1, 3-glucans binding protein (LGBP), and several serine protease zymogen involved in the proteinase cascade have been well studied (Cerenius and Söderhäll, 2004). In the crayfish *P. leniusculus*, proPO is mainly synthesized in granular cells (Gcs) and stored in the secretory granules, similar to the clotting system of horseshoe crabs (Theopold *et al*., 2004), and then released into hemocoel by exocytosis triggered by PRPs,. After binding of the PRP ligand with microbial components, serine proteinases zymogens was activated in the proPO-system, and the final step in this process is the conversion of proPO into active phenoloxidase (PO) by the prophenoloxidase activating enzyme (ppA) (Aspán *et al*., 1995). The first primary structure of proPO cDNA was reported from crayfish (Aspán *et al.,* 1995), and after that more than 40 proPO sequences have been reported from different invertebrates, such as insects, echinoderms, ascidians, bivalves, mollusks, millipedes, and brachiopods (Cerenius and Söderhäll, 2004; Cerenius *et al.,* 2008). PO is a copper-containing proteins, customarily is classified as tyrosinase, but actually arthropod POs are much more similar to arthropod hemocyanins (Burmester, 2001; Terwilliger and Ryan, 2006). Hemocyanin is a respiratory protein localized in the plasma and normally produced by the hepatopancreas in many invertebrates, and under certain conditions it exhibits PO activity and may also play important roles in immune defense against invading microorganisms (Baird *et al*., 2007; Decker and Rimke, 1998; Jaenicke and Decker, 2008; Jiang *et al*., 2007; Lee *et al*., 2004; Nagai *et al*., 2001). For instance, crayfish hemocyanin subunit 2 exhibits phenoloxidase activity after the cleavage of at the N-terminal part with trypsin, although its PO activity is relatively lower (Lee *et al.,* 2004). In the process of melanin formation, PO catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity), followed by several intermediate steps which result in the formation of melanin, a dark brown pigment.

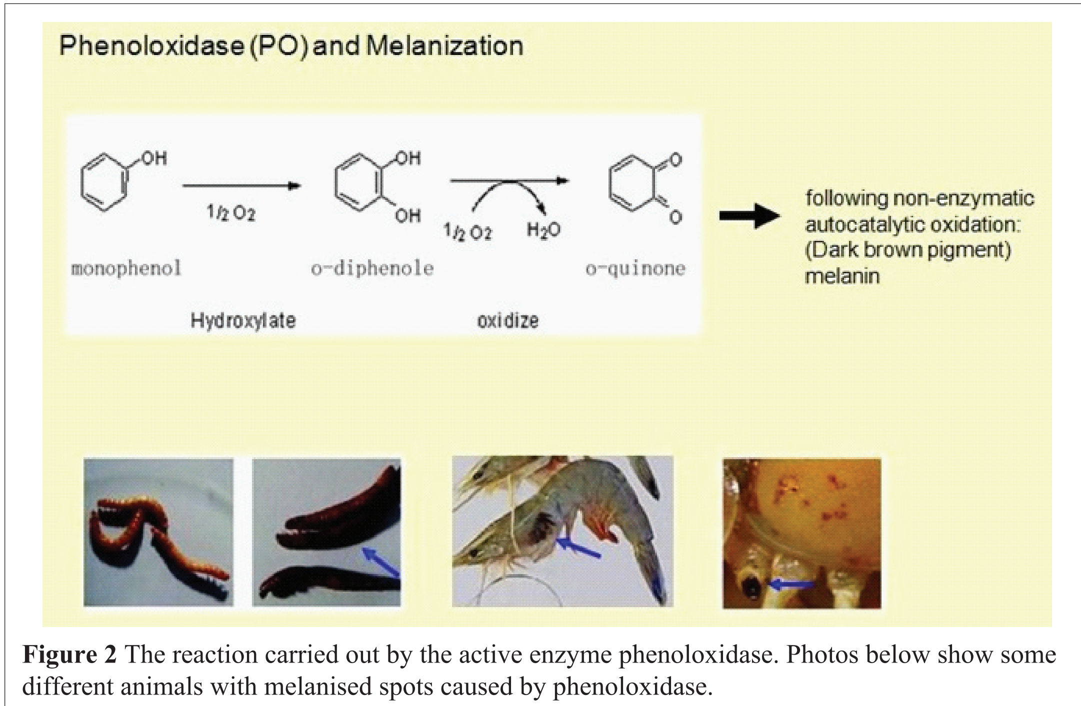

Figure 2 The reaction carried out by the active enzyme phenoloxidase. Photos below show some different animals with melanised spots caused by phenoloxidase.

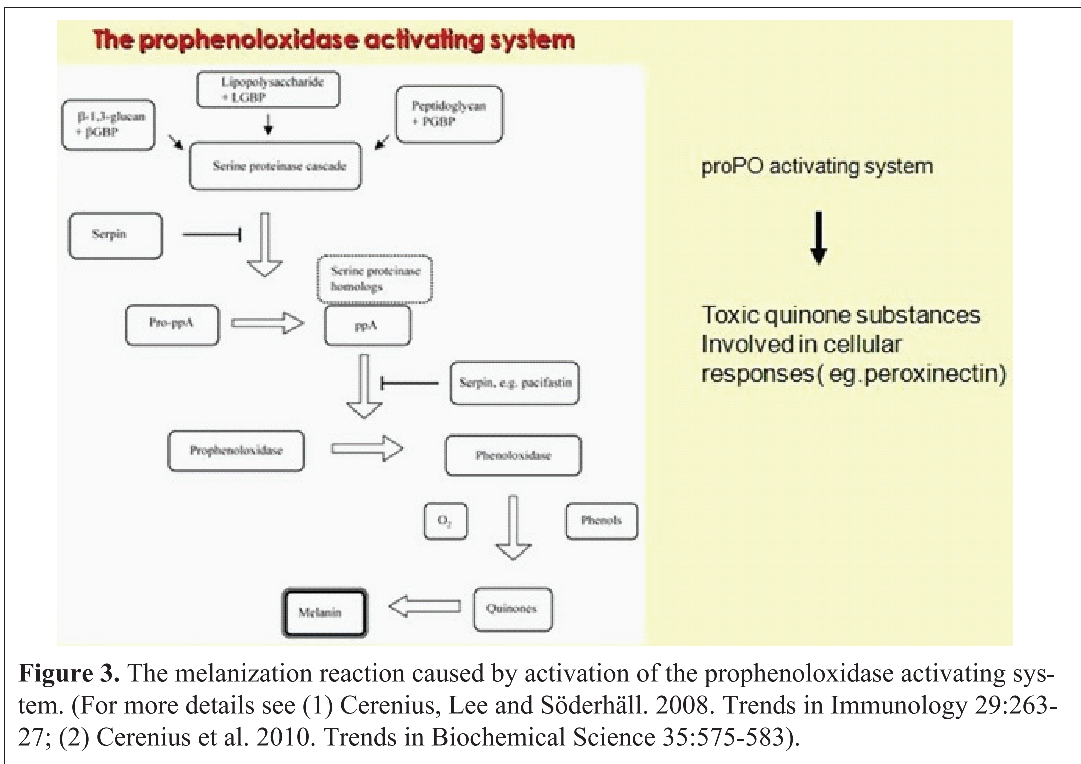

Figure 3. The melanization reaction caused by activation of the prophenoloxidase activating system. (For more details see (1) Cerenius, Lee and Söderhäll. 2008. Trends in Immunology 29:263-27; (2) Cerenius et al. 2010. Trends in Biochemical Science 35:575-583).

Crayfish ppA has similar amino acid sequence as horseshoe crab defensin (Saito *et al*., 1995). The recombinant clip domain of the endogenous trypsin-like serine proteinase ppA from crayfish accordingly shows antibacterial activity *in vitro* against Gram-positive bacteria suggesting a multiple function of crayfish ppA, which is possible for other ppAs (Wang *et al*., 2001). Several ppAs and cofactors have been identified from insects: *Manduca sexta*, *Holotrichia diomphalia*, and *Bombyx mori*, (Cerenius and Söderhäll, 2004). Crayfish and *Bombyx mori* ppAs alone can produce an active PO (Wang *et al*., 2001; Satoch *et al*., 1999), but *Manduca sexta* or *Holotrichia diomphalia* ppA need a cofactor to generate active PO (Jiang *et al*., 1998, 2003). The primary structures of these proteins show that they all exist as zymogens of typical serine proteinases, which are similar to *Drosophila* serine proteinases involved in the organization of the developing embryo (Jiang and Kanost, 2000). Masquerade-like proteins and serine proteinase homologues, other proPO activating factors, have an essential role in activation of the proPO-system in invertebrates. These proteins have no proteinase activity since the primary structures of them show a serine proteinase domain lacking the catalytic triad residues which is necessary for serine proteinase activity. This kind of proteins have been characterized from the coleopteran insects *H. diomphalia* and *Tenebrio molitor* (Kwon *et al.*, 2000; Lee *et al*., 2002; Piao *et al*., 2005), the crayfish *P. leniusculus* (masquerade-like protein *Pl*MasI) (Lee and Söderhäll, 2001), the *D. melanogaster* (masquerade protein) (Murugasu-Oei *et al*., 1995), horseshoe crab (factor D) (Kawabata *et al*., 1996) and mosquito *Anopheles gambiae* (infection-responsive serine proteinase-like protein (ispl5)) (Dimopoulos *et al*., 1997). Recently, many details have continued to be collected on the activation and the regulation of the proPO activating proteinase cascade about PRPs, serine proteinase zymogens and serine proteinase homologues. For example, hemolymph serine proteinase, HP21 was proved to act as an activator of proPO-activating proteinase 3 (PAP-3) in *Manduca sexta* (Gorman *et al.,* 2007); One synthetic Lys-PGN derivative fragment was identified as an inhibitor of the melanization cascade, and helped the understanding of how the melanization is regulated and controlled in *Tenebrio molitor* (Park *et al*., 2006); Clustering of PGRP-SA is required to activate both Toll and proPO pathways in *Tenebrio molitor* (Park *et al*., 2007). In *Holotrichia diomphalia,* the activating mechanism of proPO-activating enzyme I (PPAF I) and PPAF II was enlightened by analyzing the crystal structure (Piao *et al*., 2007; Piao *et al*., 2005). Melanin, with a strong efficiently bactericidal effect, was produced on the surface of bacteria by an active melanization complex formed with *Tenebrio* proPO and *Tenebrio* clip-domain SPH1 zymogen after both of them were converted to active forms by *Tenebrio* Spätzle processing enzyme (Kan *et al*., 2008). Zou *et al.* (2008) also found that RUNT-related transcription factor 4 regulated proPO gene expression by binding to the promoter of mosquito proPO under the control of Toll pathway in *Aedes aegypti*. All these results indicate that the two major innate immune responses, Toll pathway and the melanization reaction, share a common serine protease for their regulation (Kan *et al*., 2008).

Evidence (Liu *et al*., 2007; Johansson and Söderhäll, 1995) shows that the activation of the proPO-system can also trigger cellular responses including blood-cell adhesion, degranulation, phagocytosis, nodule formation, and encapsulation. In *Tenebrio molitor,* PO participated in cell adhesion reaction and/or clumping after binding to the hemocytes

membrane (Lee *et al*., 1999). There are reports showing that proPO binds to the surface of some hemocytes resulting in a strict spatial localization of the melanization response in *Manduca sexta* (Ling and Yu, 2005; Mavrouli *et al*., 2005). Studies from *Anopheles gambiae* indicate that PO activity is required for coagulation by causing lipophorin particles to coalesce into the sheet structures (Agianian *et al*., 2007), which increase the efficiency of hemolymph coagulation and cellular defense reactions. Some studies fully imply that the components of proPO activating system provides factors (also include the factors associated with the proPO activating system) to stimulate cellular defense through increasing phagocytosis (Cerenius *et al*., 2008). Other molecules can also gain their function when they are generated concomitant with the activation of proPO-system, for instance, peroxinectin, a cell adhesion protein from crayfish and shrimp is stored in the secretory granules of the SGCs and the GCs, which can be released during exocytosis and proPO-system activation to mediate cell attachment and spreading. (Johansson, 1999; Lin *et al*., 2007; Johansson and Söderhäll, 1988; Sritunyalucksana *et al*., 2001). By RNA interference (RNAi) of pacifastin, an efficient inhibitor of the crayfish proPO activation cascade, higher PO activity was determined, which led to lower bacterial growth, increased phagocytosis, increased nodule formation, and delayed mortality. On the contrary, silencing of crayfish proPO resulted in increased bacterial growth, lower phagocytosis, lower PO activity, lower nodule formation, and higher mortality when infected with this bacterium (Liu *et al*., 2007). The crayfish PO studies suggest that PO play a critical role in crayfish *P. leniusculus* defense when it was infected with a highly pathogenic bacterium, *Aeromonas hydrophila*.

Although melanin formation is essential for host defence in crustaceans and insects, the initiation of proPO system needs to be tightly regulated due to the danger to the animal of unwanted production of quinone intermediates and melanization in places where it is not appropriate (Ashida and Brey, 1998; Gillespie *et al*., 1997). Pacifastin is highly efficient in inhibiting the crayfish ppA, and forms a new family of proteinase inhibitors, which was named as pacifastin-like serine proteinase inhibitors, and it can inhibit the proPO system in many insects (Simonet *et al*., 2002; Vanden Broeck *et al*., 1998). Pacifastin is a high molecular weight and heterodimeric inhibitor composing of one light chain containing nine proteinase inhibitor subunits and a heavy chain that contains three transferrin lobes (Liang *et al*., 1997). Challenging crayfish with *Aeromonas hydrophila* always resulted in 100% mortality when pacifastin was silenced, indicating that endogenous proteinase inhibitors might participated into the protection of host cells or tissues by inhibiting the production of inappropriate highly toxic productions (Liu *et al*., 2007). Injection of low amount of this bacterium killed crayfish anyway in the end, which means that the *A. hydrophila* or other bacteria adopt some strategies to overcome host defenses (Vallet-Gely *et al*., 2008). By using the genetic studies, several genes expressing serine proteinase inhibitor (serpin) were shown to be involved in the melanization response toward different pathogens in *Anopheles* mosquitoes (Michel *et al*., 2005) and *Drosophila* (Ligoxygakis *et al*., 2002). It was shown that *Drosophila* Serpin 27A can inhibit its proPO activating enzyme, PPAE and prevented the melanin synthesis induced by the proPO-system (De Gregorio *et al*., 2002; Ligoxygakis *et al*., 2002). Mutations in Serpin 27A led to severe melanization and increased lethality (De Gregorio *et al.,* 2002). Injections of Serpin 27A into serpin mutant flies blocked the

melanization response on cuticle, when it is experimental injury. The survival rate of parasitoid wasp *Leptolinina boulardi* in these flies increased by the injections of Serpin27A into *Drosophila* larvae (Nappi *et al*., 2005). RNAi and loss-of-function mutation studies show Serpin 28D, one of 29 serpins in *Drosophila,* is the most strongly induced upon injury, and it regulates hemolymph PO activity in both larvae and adults. The study claims that Spn27A confines the melanin produced at the wound site, while Spn28D rather limits PO availability by controlling its initial release (Scherfer *et al*., 2008). The expression of SRPN6, another member of the serpin family, is induced by *Escherichia coli* and both rodent and human malaria parasites, and depletion of this inhibitor by RNAi delays the lysis of parasite without changing the number of developing parasites in *An. Gambiae,* indicating that SRPN6 acts on parasite clearance by inhibiting melanization and promoting parasite lysis (Abraham *et al*., 2005). *Microplitis demolitor* bracovirus carried by the wasp *M. demolitor* encodes a protein Egf1.0, which was recently shown to disable melanization reaction in *Manduca sexta* by inhibiting both the activity of PAP3 and also prevented processing of pro-phenoloxidase serine proteinase homolog (SPH) 1, and SPH2 (Beck and Strand, 2007; Lu *et al*., 2008). In addition to inhibitors of the proPO system activation, some PO inhibitor directly inhibiting the activity of PO have been studied from different arthropods (Daquinag *et al*., 1995; Daquinag *et al*., 1999; Sugumaran and Nellaiappan, 2000), and some specific pathogens have also developed strategies to prevent proPO activation: many endogenous factors function as competitive inhibitors of PO activity; several microbial derived factors also have been identified with the capacities to interfere with active PO (Daquinag *et al*., 1995; Eleftherianos *et al*., 2007; Zhao *et al*., 2005; Cerenius *et al*., 2008). For instance the phenoloxidase inhibitors (POIs), basic lysine rich peptides, have been found in *Musca domestica* and homologous structures were also detected in *Anopheles gambiae* and *Manduca sexta* (Shi *et al*., 2006; Lu and Jiang, 2007). Moreover, Volz *et al.* (2005, 2006) showed that several clip-domain serine proteases are involved in limiting parasite numbers and/or affecting the regulation of melanization. Interestingly, the PO-induced melanization reaction was found to be strictly regulated by *Tenebrio* proPO, which functions as a competitive inhibitor for the formation of melanization complex (Kan *et al*., 2008).

Melanization is vital for wound healing as well as an immune defense. Since the toxic quinone substances and by-products (intermediates) are also harmful to the host, it is necessary to tightly control the melanization reaction.

Recently, Zhao *et al*. (2005) have discovered a novel 43 kDa protein from the hemolymph of the beetle *T. molitor* (*Tenebrio* MIP) which acts as a negative regulator of melanin synthesis. This protein, the target of which is still presently unknown, is consumed during melanization, and no similarity was found between *Tenebrio* MIP and any other known protein (Zhao *et al.,* 2005). Interestingly, the *Tenebrio* MIP-antibody recognizes a protein in crayfish plasma. Since this crayfish 43 kDa protein also is involved in regulating the proPO-system and melanization, we named it *Pacifastacus leniusculus* melanization inhibiting protein (*Pl*-MIP). The sequence analysis shows that *Pl*-MIP is totally different from the *Tm*-MIP, and instead it contains of a C-terminal domain similar to vertebrate fibrinogens, but the collagenous domain found in ficolins is missing in the *Pl*-MIP corresponding region. Importantly, fibrinogen-like domain is the substrate-recognition domain of vertebrate

L-ficolins known as activators of vertebrate complement. However, crayfish MIP did not show any hemagglutinating activity as is common for the vertebrate ficolins. The structural similarities of *Pl*-MIP with ficolins indicates interesting parallels in the regulation between proteolytic cascades involved in defence in vertebrates and invertebrates. We also analysed the expression pattern in different tissue by RT-PCR. The result shows that hemocytes and Hpt did not express *Pl*-MIP. This transcript was detected at fairly low level in hepatopancreas and eyestalk, whereas high expression occurred in nerve-tissue, heart and intestine. When recombinant *Pl*-MIP (*Pl*-rMIP) was added to HLS containing an inactive proPO systeim, *Pl*-rMIP could inhibit LPS-PGN or laminarin induced PO-activity in a dose dependent manner assayed with L-Dopa as substrates, whereas no such inhibition was achieved when the proPO was activated prior to incubation with the *Pl*-rMIP. Therefore *Pl*-MIP has two functions. One is to inhibit proPO activation and the other is to block or delay melanin formation, once PO is activated. Surprisingly, an Asp-rich region similar to that found in ficolins that is likely to be involved in Ca^{2+}-binding is present in crayfish MIP. The antigenic Asp-rich region is important for the function of *Pl*-MIP, since site-directed mutagenesis was perfomed by deleting the four-Asp amino acids in the recombinant protein and this mutant form of MIP acids lost its original function, which implicates that MIP is involved in regulating the PO activating cascade.

The sequence similarity between *Pl*-MIP and human L-ficolin was used to build a homology model of *Pl*-MIP 3-D structure. The model suggests that *Pl*-MIP is an alpha/beta protein stabilized by two cysteine bridges. The binding site for Ca^{2+} ions as described in the L-ficolin structure and similar to tachylectin 5A is well conserved in *Pl*-MIP.

CONCLUSION

Altogether, we have identified three different proteins which can be used as markers for Hpt cells, SGCs and GCs of *P. leniusculus* respectively, and the newly described hemocyte lineage marker protein genes from crayfish *P. leniusculus* provide more information about the differentiation of different stages of the crayfish hemocytes. These data taken together may be helpful for future studies to reveal the connection between SGCs, GCs, and precursor cells in Hpt and also the role of the growth factors (e.g. astakine) as regulators of hemocyte maturation and development in crustaceans.

In the second study, we have identified a novel protein that acts as a negative regulator of proPO activation and melanization. Our data shows that the *Pl*-MIP is most likely an important regulator of the proPO-system and will keep the proPO-system in a non active form until certain inducers such as pathogen-associated molecular patterns (PAMPs) or microorganisms are present, then *Pl*-MIP as well as *Tenebrio* MIP (Zhao *et al*., 2005) are specifically degraded which then will allow activation of the proPO-sytem and melanization. Altogether, both crayfish MIP as well as *Tenebrio* MIP do not affect phenoloxidase activity in itself, but instead interfere with the melanization reaction from quinone compounds to melanin, which is different from Pacifastin, a highly efficient inhibitor of the crayfish ppA.

This study suggests that there may be more unknown factors involved in regulating the proPO system, and the mechanism of *Pl* MIP or *Tenebrio* MIP degradation in the proPO activation need further studies.

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Toll-like receptors in teleosts

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ABSTRACT

Toll-like receptors (TLR) recognize the pathogen-associated molecular patterns (PAMPs) and regulate the subsequent immune responses in mammals. We have so far succeeded in finding the 11 types of TLR genes from Japanese flounder (*Paralichthys olivaceus*). TLR genes have also been found in the genomes of fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*). However, only limited information is known about the function of TLRs in fish. The fragmentary information of the fish TLR gene expression, immune response following PAMPs stimulation and intracellular signaling are available in fish. In addition, the phylogenetic analysis in vertebrate TLR genes and comparison of TLR family members between teleost fish species revealed that specific divergence of TLR genes between mammals and fish as well as between fish species. Here, the possible functions of teleost fish TLR and its importance are discussed.

Key words: immune response; pathogen molecular pattern (PAMP); toll-like receptor (TLR).

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INTRODUCTION

The innate immune system is responsible for the first line of defense in host protection against invading microbial pathogens and it is relatively evolutionarily conserved among mammals (Akira *et al*., 2006). The pathogen-associated molecular patterns (PAMPs) initiate the host innate immune responses of animals including insects and vertebrates. *Drosophila* Toll, which was initially identified as a receptor essential for dorso-ventral polarity during development. This Toll was first shown to participate in innate immune responses against fungal infections in 1996 (Lemaire *et al*., 1996). Subsequently, numerous homologues of Toll, termed Toll-like receptors (TLRs), were identified in mammals. These receptors were also demonstrated to recognize PAMPs to trigger the networks of innate immune responses (Akira *et al*., 2006). Mammalian TLR signaling pathways which are triggered by different TLRs and consequent immune responses are highly distinct and diverged. To date, two major TLR signaling pathways of MyD88-dependent and -independent pathways were known. The MyD88-dependent pathway leads to activation of AP-1 transcription factors through activation of MAP kinases and NF-κB activation through activation of the IKK complex. Activation of these transcription factors triggers inflammatory cytokine production (Takeda and Akira, 2005). The MyD88-independent pathway is transduced from TLR3 and TLR4. This pathway activates interferon (IFN) regulatory factor 3 (IRF3) with consequent production of IFN-β (Takeda and Akira, 2005). The complex of these signaling pathways modulate the proper immune response against pathogens..

The first report of fish TLR gene was found in gold fish, *Carassius auratus auratus* (Stafford *et al*., 2003). The conservation of essential domain structures in vertebrate TLRs, including leucine-rich repeats (LRRs) in the extracellular portion and a Toll/IL-1 receptor homology (TIR) domain were confirmed in goldfish TLR. In a same year, TLR genes have been globally surveyed from the draft whole genome sequence of fugu (*Takifugu rubripes*) and unique TLR genes were reported (Oshiumi *et al*., 2003). In addition, zebrafish (*Danio rerio*) genome database was also surveyed to discover the all of predictable TLR genes and reported in 2004 (Jault *et al*., 2004; Meijer *et al*., 2004). In our study Japanese flounder (*Paralichthys olivaceus*), we have identified 11 types of TLRs homologues. These results were reported at the 7th Symposium on Diseases in Asian Aquaculture. To date, the involvement of teleost fish TLRs in the innate immune system have been uncovered by virtue of TLR gene cloning and their fundamental studies. In addition, several novel TLR genes were discovered in teleost fish. Hence, the immune response which is introduced in the PAMPs recognition through the fish TLRs will be an intrigue topic for the comparative immunology. Here, to clarify the future direction of study in teleost fish TLRs, the present information of teleost fish TLRs are briefly summarized and their possible functions are discussed.

1. Structural conservation of TLRs between teleost fish and mammal

TLRs are single-pass transmembrane proteins composed of an N-terminal extracellular LRR domain and C-terminal intracellular domain of TIR domain. This basic structure is highly conserved in TLRs of animal. The LRRs are responsible for recognition of respective ligand

of pathogen components. The extracellular domains of TLRs contain about 20 to 30 LRRs sandwiched between the LRR N-terminal (LRRNT) domain and LRR C-terminal (LRRCT) domain modules (Matsushima *et al*., 2007) to shield the bydrophobic core of the first LRR at the N-treminal or last LRR at the C-terminal (Matsushima *et al*., 2005; Kajava, 1998). A LRR is consisted of 20 to 33 amino acid residues. The 'LXXLXLXXN' elements are conserved in the LRR (Jin and Lee, 2008; Matsushima *et al*., 2007). Bell *et al*. (2003) suggested that the variable sequence and length of insertion is contained at the following position of 10 and 15 amino acid residue in the LRR consensus sequence to generate the variety function of TLRs. A LRR is known to form a arc-shape structure, then juxtaposition of LRR loops produces a solenoid-like structure. Today, the variation of the solenoid-like structure in extracellular domain which is generated from the different LRR conformations is considered to be related to the specific PAMPs recognition of TLRs (Bell *et al*., 2003). Interestingly, a certain level of conservation in LRR sequence between the same class of TLRs in teleost and mammal are reported, including a class of TLR5 and a class of TLR9 (Takano *et al*., 2007; Tsoi *et al*., 2006). Hence, these structural conservation may indicate the recognition of the common PAMP between fish and mammalian TLRs.

Intracellular TIR domain of TLR initiates the signal transduction to associate with another TIR domain of adaptor proteins. The TIR domain is composed of roughly 200 amino acid residues in length. The three functionally important regions box 1, box 2 and box 3 are conserved in mammalian TLRs (O'Neill, 2000; Rock *et al*., 1998). The involvement of these three boxes in signal transduction were confirmed by mutagenesis studies of human, *Homo sapiens* (Slack *et al*., 2000). The conservation of these boxes have also been reported from teleost fish TLRs (Jault *et al*., 2004; Takano *et al*., 2007). The assessment of fugu, zebrafish and mammalian TIR domain evolution (Meijer *et al*. 2004) suggested coincidental evolution among the same class of TLRs even between fish and mammals. "Coincidental evolution" is a term describing phylogenies with branches that do not evolve independently (Roach *et al*. 2005). Favor interpretation for this phenomena is the conservation of similar TLR signaling cascade in fish. In fact, the conserved homologues of four of the TIR domain adaptor proteins (MyD88, MAL, TRIF and SARM) from zebrafish and Fugu have been reported (Meijer *et al*., 2004). These reports clearly show that the TLR system has an important role in fish immunity.

2. TLR repertoire

Today, a total of 10 TLRs numbered 1-10 in humans and a total of 13 TLRs in mouse (*Mus musculus*) have been identified (Verstak *et al*., 2007; Akira, 2004). TLR1-9 are conserved in humans and mice. TLR10 is functinal in humans but mouseTLR10 gene is disrupted by two retroelements. On the other hand, human TLR genes corresponding mouse TLR11-13 are represented only by a pseudogene (Roach *et al*., 2005).

At the beginning of this decade, the TLR family are surveyed from draft genome sequence of teleost fish, such as fugu (Oshiumi *et al*., 2003) and zebrafish (Jault *et al*., 2004). Surprisingly, higher numbers of diverged TLR than mammalian TLR genes are discovered from these fish including novel TLR genes. TLR1, -2, -3, -5, -7, -8 and -9 genes

were conserved in the both fish as well as mammal, while mammalian TLR4 homologue was only seen in zebrafish genome. In addition, TLR1 family of TLR6 and TLR10 genes are missing in the teleost fish (Roach *et al*., 2005). For the novel TLR genes, TLR14, 21 (also found from aves), 22 and 23 including soluble form TLR5 (also found from amphibian) were identified from the fugu, while TLR18 (orthologue of fugu TLR14), 19, 20, 21 and 22 gene have been identified from zebrafish (Oshiumi *et al*., 2003; Jault *et al*., 2004; Meijer *et al*., 2004; Roach *et al*., 2005). The duplication of zebrafish TLR4, 5, 8 and 20 gene were reported by Meijer *et al*. (2003). From the comparison of TLR gene loci between fugu and zebrafish using the ensemble genome browser (http://oct2007.archive.ensembl.org/index.html), the tandem duplication of zebrafish TLR19 (gene ENSDARG00000026663 and ENSDARG00000070392) was also evident. However, it is seems that the tandem gene duplication of many TLR genes have been happened only in zebrafish genome but not in fugu genome. Further, interspecific specific TLR gene duplication was reported from rainbow trout (*Onchorhynchus mykiss*) TLR22a and TLR22b showing 95.6 % homology in nucleotide sequence level (Rebl *et al*., 2007).

Taken together, TLR repertoire, such as TLR1, 2, 3, 5, 7, 8 and 9, between teleost fish and other vertebrates seems to be highly conserved. Because, it is almost certainly that microbes cannot mutate basic structure of their PAMPs (Roach *et al*. 2005), and these PAMPs have became the essential ligands for the TLRs. On the other hand, the novel TLR gene in fish and species specific TLR gene divergence is becoming clear. This difference is favorably interpreted as a result of different selection pressure of exposed pathogens in distinct environments (Oshiumi *et al*. 2003). Therefore, comparison of immune responses which are mediated through different repertoire TLRs between human and teleost fish species are interesting as well as comparing between teleost fish species.

3. Toll-like receptor of teleost fish and immune response against PAMPs

The ligands for mammalian TLRs are reviewed by Kawai and Akira (2007). The study on specific ligands determination for the teleost TLRs are limited. However, as mention above, there is conservation of LRR structure between teleost fish and mammalian TLR. Therefore, teleost fish TLR may be able to recognize the similar PAMPs with mammalian TLRs. Here, the immune responses against PAMPs stimulation and possible corresponding TLRs of teleost fish is discussed.

3.1. TLR1 and TLR2

TLR1 and TLR2 gene are commonly existed in the teleost fish and it may indicate functional importance for immune modulation. In mammal, TLR1 and TLR6 is able to associate with TLR2 to form a heterophilic dimer and discriminate the lipid structures between diacylated and triacylated lipoproteins, respectively. (Takeda and Akira, 2005). As mention above, TLR1 family of TLR6 and TLR10 does not existed in teleost fish. Therefore, it is intrigue that whether teleost fish are able to discriminate the two types of lipopeptide. Purcell and his colleague conducted the diacylated lipoprotein (Pam2CSK4) and triacylated lipopeptide

(Pam3CSK4) stimulation of rainbow trout leukocytes (Purcell *et al*., 2006). Interestingly, the transcription level of IFN-α1 and IL-1β 1 were increased in the both diacylated and triacylated lipoprotein stimulated leukocytes. But, it is still unclear whether both lipoproteins are recognized by TLR1/TLR2 heterophilic dimer or functional substitute molecules in teleost fish.

Mammalian TLR2 is also able to recognizes Gram-positive bacteria cell wall component of peptidoglycan (PG). The PG is also known as a strong inducer of innate immune response in teleost fish. We demonstrated the TLR2 gene up-regulation and increased number of TLR2 expressing leukocytes in Japanese flounder following PG stimulation. The PG induces significant production of cytokines such as IL6-cytokine subfamily that are involving cellular development, inflammatory function, and acute phase and immune responses have been reported in teleost fish (Hwang *et al*., 2007; Castellana *et al*., 2008). These reports may support the functional involvement of TLR2 to the PG recognition of fish immune system as well as mammalian TLR2.

3.2. TLR3

In mammal, pathogen derived double stranded RNA such as RNA virus genome are recognized by TLR3. The double stranded RNA (dsRNA) analogue of poly I:C have been used as antiviral immune response mediator in teleost fish. The injection of poly I:C to Atlantic salmon (*Salmo salar* L.) resulted gene up-regulation of type I interferon (IFN) and antiviral Mx gene in spleen and kidney (Strandskog *et al*., 2008). Further, the gene expression of rainbow trout IFN regulatory factor (IRF) 3 having crucial role in TLR3 signaling were up-regulated following poly I:C stimulation in leukocytes (Holland *et al*., 2008). Therefore, TLR3 mediated immune responses in teleost fish may have an important role in viral infection as well as that of mammals. Recently, two intracellular dsRNA receptor of retinoid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) were reported in teleost fish (Robertsen, 2008). The functional corporation of TLR3 and these intracellular dsRNA receptor may also be important for type I IFN production in teleost fish.

3.3. TLR4

TLR4 is known as a receptor for bacterial lipopolysaccharide (LPS) (Kawai and Akira, 2007). Zebrafish has two TLR4 genes, but there are no report of TLR4 molecules from other fish species. LPS have been widely used as immunostimulant of teleost fish and numbers of study demonstrated the potential of LPS for mediating pro-inflammatory cytokine production, chemokine production, nitric oxide production and macrophage activation (Neumann *et al*., 1995; Stafford *et al*., 1999; Corripio-Miyar *et al*., 2007; Darawiroj *et al*., 2008). It is highly possible that there are substitution of TLR4 in teleost fish for sensing LPS. Whilst, the possibility of the immunostimulation effect by the contaminant in the crude LPS preparation was mentioned from the recent study of rainbow trout (Purcell *et al*. 2006). However, there are no other available studies to elucidate these discrepancy.

3.4. TLR5

TLR5 is responsible for the bacterial flagellin recognition. The unique point of teleost fish TLR system as well as amphibian is existence of both membrane type TLR5 (TLR5M) and soluble form TLR5 (TLR5S) which do not contain C-terminal intracellular domain of TIR and transmembrane domain (Tsujita *et al*., 2005). The both rainbow trout TLR5 are able to recognize the flagellin of *Vibrio anguillarum*. Rainbow trout TLR5S gene is predominantly expressed in the liver and highly up-regulated following *V. anguillarum* stimulation in the rainbow trout hepatoma cell line, similar as acute phase proteins produced from the liver. The activity of NF-κB occurring from TLR5M signaling was potentiated by the combination of rainbow trout TLR5M and TLR5S. From this observation, Tsujita *et al*. (2005) hypothesized that the combination of TLR5M and induced circulatory TLR5S systemically provoke robust activation of NF-B, which leads to full response to flagellin in the whole body. To date, TLR5S genes were found from several fish species (Oshiumi *et al*., 2003; Baoprasertkul *et al*., 2007). This unique flagellin recognition system is seems to be conserved in the wide range of the teleost fish. On the other hand, TLR5S have not been found form zebrafish, so comparison of sensitivity against flagellin between zebrafish and other teleost fish TLR5 is interesting topic for the further study.

3.5. TLR7, 8 and 9

Mammalian TLR7, 8 and 9 detect single stranded nucleic acids derived from pathogens in the endosome. Especially, TLR7 recognizes single stranded RNA derived from various viruses as well as synthetic imidazoquinolines. The human TLR8 also participates in the recognition of ssRNA and imidazoquinolines, whereas the function of mouse TLR8 remains unclear (Kawai and Akira, 2007). Immidazoquinolines are also efficient immune modulator in teleost fish. Imidazoquinolines such as S-27609 and R848 are reported as antiviral immune mediator of salmon, although he corresponding receptor either TLR7 or TLR8 have not been defied (Purcell *et al*., 2006; Kileng *et al*., 2008; Sun *et al*., 2008). The drastic gene up-regulation of IFN-α1, IL-1β IL-8, TNF-α1 and α2 were occurred following R848 stimulation in rainbow trout leukocytes, whereas the leukocytes did not respond to another compound of imidazoquinoline of loxoribine (Purcell *et al*., 2006). IFNa (two genes), IFNb (four genes) and IFNc (five genes) were found in Atlantic salmon (Sun *et al*., 2008). To study whether these different salmon IFN subtypes are associated with imidazoquinoline specific IFN induction pathways, the expression pattern of these genes were profiled following S-27609 (Sun *et al*, 2008). Interestingly, only IFNb genes were strongly up-regulated by S-27607, and weakly up-regulated by poly I:C in Atlantic salmon. From this observation, the author speculated that the IFNb genes are mainly induced through the TLR7 or TLR8 pathway whilst other IFN genes are induced through the TLR3, RIG-I and/or MDA-5 pathway. Taken together, it is likely that the salmonid TLRs responsible for single stranded RNA is able to discriminate the structure of imidazoquinolines and activate specific signaling pathway to produce particular cytokines such as Atlantic salmon IFNb genes.

Another nucleic acids receptor of TLR9 recognizes unmethylated CpG DNA motifs present in bacterial and viral genomes. The CpG oligodeoxynucleotides (ODN) mimicking unmethylated CpG DNA have been used for the studies on vertebrate TLR9 studies including

teleost fish. Takano *et al*. (2007) demonstrated specific recognition of CpG ODN by TLR9 and activation of TNF-α gene promoter following CpG ODN stimulation in Japanese flounder. Further, more than twenty kind of CpG ODNs were assessed to confirm optical motif for immunostimulation of Atlantic salmon by measuring kidney leukocytes IFN-like activity, and the the highest IFN-like activity were observed following CpG ODN 1681 stimulation (Jørgensen *et al*., 2003). In addition, the uptake of CpG ODN by head kidney leukocytes (macrophages) and enhancement of the type I IFN and Mx production following CpG ODN 1681 were confirmed in Atlantic salmon (Pedersen *et al*., 2006). Hence, teleost fish TLR9 have pivotal role in the CpG DNA recognition and there is a optical CpG DNA motif for the antiviral activity induction such as IFN production.

3.6. Novel TLRs

The TLR14 subfamily expanded in teleost fish and amphibians. For the function of TLR14, the favorable hypothesis was mentioned by Roach *et al*. (2005) based on the analysis of TLR evolution in vertebrates. The phylogenetic analysis of TLR genes revealed assortative evolution of TLR14 and the lipopeptides specific TLRs including vertebrates TLR1, -2, -6 and -10. The TLR6 and TLR10 homologue in teleost fish have not been found, while TLR14 have not been reported from mammals. Therefore, teleost fish TLR14 was speculated as functional substitute for mammalian TLR6 and TLR10. Recently, the functional involvement for the long double stranded RNA recognition by fugu TLR22 was reported. Hence, the fugu TLR22 is said as a functional substitute of human cell-surface TLR3 (Matsuo *et al*., 2008). Meanwhile, the functional information of TLR19, -20, -21 and -23 are completely lacking and further studies on these novel TLRs are eagerly awaited.

4. Future perspectives

4.1 Functional comparison and characterization of TLR systems in teleost fish

It is evident that numerous molecules involved in biodefense system are resemble and are conserved in mammals and teleost fish. Here, the high level of conservation of TLRs between fish and mammals have been discussed and it is almost certain that PAMPs recognition by TLR is one of the crucial mechanisms for innate immunity in fish. On the other hand, there are novel TLRs in teleost fish although their functions have not been identified. Interspecific TLR diversity have also been observed in the fish genome. Especially, it is said that zebrafish experienced the relativity recent TLR gene duplications (Meijer *et al*. 2004). Therefore, comparison of immune modulation from the different repertoire of TLR in different species are important. Furthermore, some of novel TLRs in teleost fish are hypothesized to compensate to the function of mammalian TLRs such as TLR4, 6 and TLR10 which have not been found in teleost fish (Oshiumi *et al*. 2003; Roach *et al*. 2005). Therefore, functional analysis in the novel fish TLRs are also interesting subject for the comparative immunology between mammals.

4.2. Analysis of immunomodulation by TLR-expressing cells

To survey for infectious agents particularly at the epithelial tissues including mucosal surfaces are interlaced with resident innate leukocytes such as dendritic cells (DCs),

macrophages and mast cells. In mammal, antigen-presenting cells (APCs) such as DCs play central role for orchestrating immune response. Especially, TLR signaling are important for the modulation of DCs activity. DCs are involved in the generation of multiple effector cell types, including TH1, cytotoxic T cell (CTL) and B cell responses (Kaisho and Akira, 2006; Iwasaki and Medzhitov, 2004). Therefore, the studies on mammalian TLR-expressing cells are prosperously conducted for the therapeutic purpose such as vaccine and its adjuvant development.

On the other hand, fish leukocytes expressing TLRs have not been classified. Transcription of fish TLR genes were highly occurring in the leukocytes (Hirono *et al*., 2004; Purcell *et al*., 2006; Takano *et al*., 2007; Holland *et al*., 2008). Further, we demonstrated the proliferation and infiltration of Japanese flounder TLR9-expressing cells at the site of bacterial infection (Takano *et al*., 2007). Thus, the fish leukocytes may also important in the modulation of immune responses. Therefore, the classification of teleost fish TLR-expressing cells and analysis of their role in immunomodulation followed by TLR signaling are also essential for the development of efficient therapeutic techniques for aquaculture.

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Identification of *Edwardsiella tarda* antigens recognized by mucosal natural antibody in olive flounder, *Paralichthys olivaceus*

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ABSTRACT

Olive flounder is an important mariculture fish species in Korea. However, the culture of this species has been threatened due to a variety of infectious agents, especially *Edwardsiella tarda*. The skin mucus of fish, which contains a higher amount of immunity related substances, acts as an innate barrier, protecting them from invasive environmental pathogens. In the present study, we investigated the ability of the natural antibodys (NAbs), secreted in the cutaneous mucus, to recognize *E. tarda* antigens using 2-dimentional gel electrophoresis (2-DE) and immunoblotting. Further, the antigenic proteins recognized by NAbs were identified using electrospray ionization tandem mass spectrometry (ESI-MS/MS) as outer membrane proteins, phosphopyruvate hydratase, phosphoglycerate kinase, Ribose-5-phosphate isomerase A, and triosephosphate isomerases. These proteins recognized by mucosal NAbs are suspected as important epitopes in *E. tarda* to produce the antigen-antibody complexes in innate immunity of mucus.

Keywords: *Edwardsiella tarda*; mucus; natural antibody; *Paralichthys olivaceus*; ESI-MS/MS

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INTRODUCTION

Olive flounder is a type of flatfish, which is regarded as a delicacy in Korea. It is the major aquaculture fish species in Korea with production reaching 54,600 metric tons in 2009 (Korea Statistical Information Service, KOSIS, http://kosis.kr). However, cultured fish are often threatened by infectious agents from the environment resulting in mass mortalities. *Edwardsiella tarda*, a Gram-negative, motile, short rod, causes edwardsiellosis in both freshwater and marine fish, and is a major bacterial pathogen of olive flounder (Thune *et al.,* 1993; Mohanty and Sahoo, 2007).

The olive flounder, which differs from most teleost fish by having a more sedentary bottom-dwelling lifestyle, has an abundance of cutaneous mucus. It is thought that the cutaneous mucus acts as an immunological barrier in these fish, in much the same way as those of the gut, respiratory tract and genital tract (Shephard, 1994; Woof and Mestecky, 2005). The mucus plays a critical role in the defense mechanism of the fish, acting as the first physiological barrier by eliminating pathogens through the continuous production of mucus (Kanno *et al.,* 1989; Ellis, 2001). Furthermore, it acts as a biological barrier because of immune substances within mucus which may include C-reactive proteins, alkaline phosphatase, protease, lysozyme, transferrin, and immunoglobulins (Igs) (Alexander and Ingram, 1992; Fast *et al.,* 2002; Palaksha *et al.,* 2008). Particularly, one of the important molecules in cutaneous mucus of fish is immunoglobulins (Igs) playing a key role in recognizing natural antigens. IgM is known to be the major immunoglobulin in the mucus of fish, which contrasts with the IgA in the mucus of mammals (Woof and Mestecky, 2005; Hatten *et al.,* 2001). The mucosal IgM has also been reported in several fishes; such as catfish, *Ictalurus punctatus* (Di Conza and Halliday, 1971; Zilberg and Klesius, 1997), carp, *Cyprinus carpio* (Rombout *et al.,* 1986) and Atlantic salmon, *Salmo salar* (Hatten *et al.,* 2001). In addition, the IgM has been suggested as a natural antibody (NAb) in olive flounder (Palaksha *et al.,* 2008). However, the knowledge of the interaction of NAbs with bacterial pathogens in flounder mucus is still unknown.

Therefore, the present study aimed to elucidate the interaction between NAb and infectious bacteria such as *E. tarda*. Utilizing a proteomics approach, common antigenic proteins were observed in *E. tarda* which were recognized by NAb of the cutaneous mucus. Subsequently, the recognized proteins were identified by nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS).

MATERIALS AND METHODS

Preparation of cutaneous mucus

Cutaneous mucus of fish ranging in size from 500-800 g was collected from an edwardsiellosis-free farm in Namhae, Gyeongnam, Korea in autumn 2008. The mucus was prepared by a method modified from Palaksha *et al.* (2008). Briefly, mucus was gathered and mixed from

10 fish by scraping with a soft rubber spatula. The mixed mucus was transferred to sterile 50ml polypropylene conical tube. The samples were transferred to the laboratory on ice and kept at -80°C. To prepare clarified mucus extract (CME); the stored mucus was mixed with 10 volumes of TBS buffer (Tris-buffered saline; 50mM Tris-HCL, pH 8.0, 150mM NaCl); and frozen and thawed repeatedly. The homogenate was centrifuged at 2850×*g* for 60min, and supernatant was filtered with 0.45μm syringe filter (Minisart). The filtered supernatant was lyophilized and dissolved in deionized water (dW, Millipore MilliQ, 18.2 MΩ cm); subsequently, the solution was replaced with TBS buffer and concentrated with Amicon YM3 membrane (Amicon, Danvers, MA). All of the steps were performed on ice or below 4°C, to minimize denaturation or digestion of proteins.

Preparation of bacteria sample

Edwardsiella tarda (ED-45) was previously isolated from Jeju, Korea in 2004. The bacterium was kept at -80°C in 10% (*v/v*) glycerol-trypticase soy broth supplemented with 2% (*w/v*) NaCl (TSB-2). The bacteria were cultured in 50mL TSB-2 at 26°C until an optical density $(OD)_{610nm}$ of 1.0, pelleted by centrifugation at 2,000×*g* for 30min at 4°C, and washed 3 times with PBS (phosphate-buffered saline; 3mM KCL, 137mM NaCl, 1.5mM KH_2PO_4, and 8mM Na_2HPO4, pH7.4). The pellet was suspended in 5ml deionized water and aliquoted to 1ml. The suspensions were sonicated (XL-2020, Misonix) at 120W for 10 min (sonication for 5s, 20s interval) in ice slurry; and centrifuged at 16,000×*g* for 30 min at 4°C. The resulting supernatant was stored at -20°C until use.

1-DE and immunoblotting

The supernatant from the sonicated bacterium was applied to one-dimensional SDS-PAGE (1-DE) according to Laemmli (1970). Ten microgram of protein was applied to two separate 12.5% (*w/v*) SDS-PAGE under denatured condition. One gel was stained with Commassie brilliant blue R-250 for visualization, the other gel was used for immunoblot assay. The proteins in the gel were transferred to PVDF membrane (Millipore, USA) at 60V for 60 min and blocked with 5% (*w/v*) skim milk in PBS-T (3mM KCL, 137mM NaCl, 1.5mM KH_2PO_4, and 8mM Na_2HPO4, pH7.4, and 0.05% (*v/v*) Tween-20) for 60min at RT. Blocked membrane was washed 3 times with PBS-T, and incubated with 200μg of CME protein in 4 mL 5% (*w/v*) skim milk in PBS-T for 2h at 26°C. The membrane was incubated with anti-flounder IgM monoclonal antibody (MAb) (Shin *et al.,* 2007) for 60 min at 26°C after washing 3 times with PBS-T, washed 5 times with PBS-T, and incubated for 60 min with goat anti-mouse IgG-HRP (Jackson. USA; 1:2,000). Finally, proteins recognized by CME were developed with an ECL kit (Enhanced Chemiluminescence; Amersham Biosciences).

2-DE and immunoblotting

Two-dimensional electrophoresis (2-DE) samples were prepared using 100μl of supernatant from sonicated bacteria, the supernatant was mixed with 400μl lysis buffer (40mM Tris-HCL, 7M urea, 2M thiourea, 4% (*w/v*) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5% (*v/v*) IPG buffer pH 3-10, and 1.4% (*w/v*) DTT) and vortexed at RT for 30 min. 200 μg of proteins from lysate were precipitated with equal volumes of 20% trichloroacetic acid. The precipitate was washed in acetone and dissolved

in 250 µl rehydration buffer (9M urea, 4% (*w/v*) CHAPS, 5% (*v/*v) IPG buffer pH 4-7, 1.4% (*w/v*) DTT, and trace amount of bromophenol blue); and vortexed for 30 min at RT then centrifuged at 16,000×*g* for 15 min. Isoelectrofocusing (IEF) and 2-DE were performed according to previous study (Shin *et al.,* 2006). Briefly, the supernatants were subjected to IEF (IPGphore™ system, Amersham Bioscience, Uppsala, Sweden) with IPG strips (Immobiline DryStrip™, pH 4-7, 13 Cm; Amersham Bioscience). The IPG strips were equilibrated with equilibration buffer (6M urea, 2% SDS (*w/v*), 30% glycerol (*v/v*), 50 mM Tris-HCL, and 0.002% bromophenol blue, pH 8.8). Equilibrated strips were electrophoresed on 12.5% SDS-PAGE gel. The gel was subjected to 2-DE immunoblotting, which was performed as described in the previous section and visualized by silver staining.

Protein identification using ESI-MS/MS

Recognized protein spots based on 2-DE immunoblotting result were excised from 2-DE gels and extracted by in-gel digestion with trypsin as described previously (Shin *et al.,* 2006). Digested peptides were applied to nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to Lee *et al.* (2004). Briefly, the supernatant of tryptic digestion were purified by a microcolumn packed with a Poros R2 resin (PerSeptive Biosystems) in GELoader tips (Eppendorf, Hamburg, Germany). Purified peptides were applied to a QSTAR pulsar-i MS system (AB/MDS Sciex, Toronto, Canada) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark) to obtain the MS/MS data. Ionspray voltage was set to a potential of 850–900 V. Peptide data were acquired from *m/z* range of over 400–1200 Da in positive mode with manually optimized collision energy settings for each peptide. The data were processed and interpreted with the BioAnalyst (PerSeptive Biosystems) software. Mascot (http://www.matrixscience.com) was employed to interpret the raw MS/MS data. The default search parameters used were enzyme = trypsin, variable modification = carbamidomethyl (C), peptide tolerance of parent ion ± 1 Da, MS/MS tolerance ±0.8 Da, and maximum missed cleavage = 1. Proteins with a Mascot score of more than 15 ($p<0.05$) was considered reliable and used in the present study. The identified peptides were aligned by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search to confirm the identity to *E. tarda* protein.

RESULTS

SDS-PAGE and immunoblotting was employed to detect proteins suspected as common antigens in *E. tarda* which can be recognized by natural antibodies in cutaneous mucus. The proteins were separated by 1-DE under denatured condition (Fig. 1a) and was used for immunoblotting. There were several weak bands in *E. tarda* 1-DE immunoblot results. CME and anti-IgM L chain (anti-IgM) MAb (Shin *et al.,* 2007) were used to detect antigenic proteins which can be regarded as specifically recognizable antigens by the natural antibody (NAb) mucus IgM (Fig. 1b). To identify the proteins recognized by mucosal NAb, proteins of *E. tarda* were separated by 2-DE and visualized by immunoblot analysis with CME and anti-IgM MAb. Around 400 spots were visualized in 2-DE of *E. tarda* under a pH range of 4-7 (Fig. 2). The 2-DE gel was transferred to PVDF membrane and applied to immunoblot analysis (Fig. 3). Sixteen spots were recognized by CME, 7 of which were then identified

by ESI-MS/MS analysis (Table 1), spot number 1 and 2 were outer membrane proteins of *E. tarda*; spot 3 was identified as phosphopyruvate hydratase from *Photobacterium profundum ss9*; spot 4 was phosphoglycerate kinase of *Edwardsiella ictaluri*; spot 5 was Ribose-5-phosphate isomerase A of *Enterobacter cloacae*; and spot 6 and 7 were two triosephosphate isomerases from *Gloeobacter violaceus PCC 7421* and *Serratia proteamaculans 568*, respectively (Table 1). The identified proteins from the database of other species were later compared to *E. tarda* aligned by BLAST search, which showed very high sequence similarity except for the 82% of *Gloeobacter violaceus PCC* 7421.

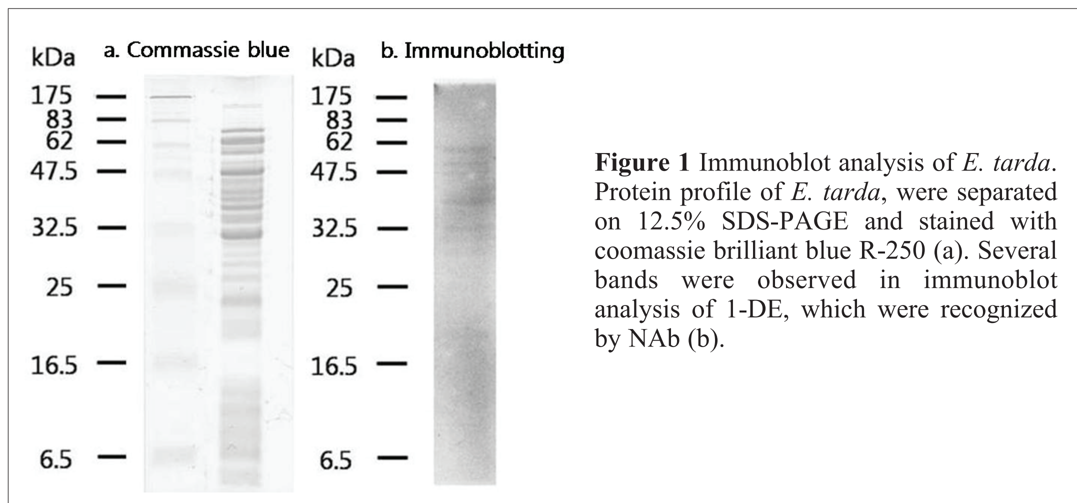

Figure 1 Immunoblot analysis of *E. tarda*. Protein profile of *E. tarda*, were separated on 12.5% SDS-PAGE and stained with coomassie brilliant blue R-250 (a). Several bands were observed in immunoblot analysis of 1-DE, which were recognized by NAb (b).

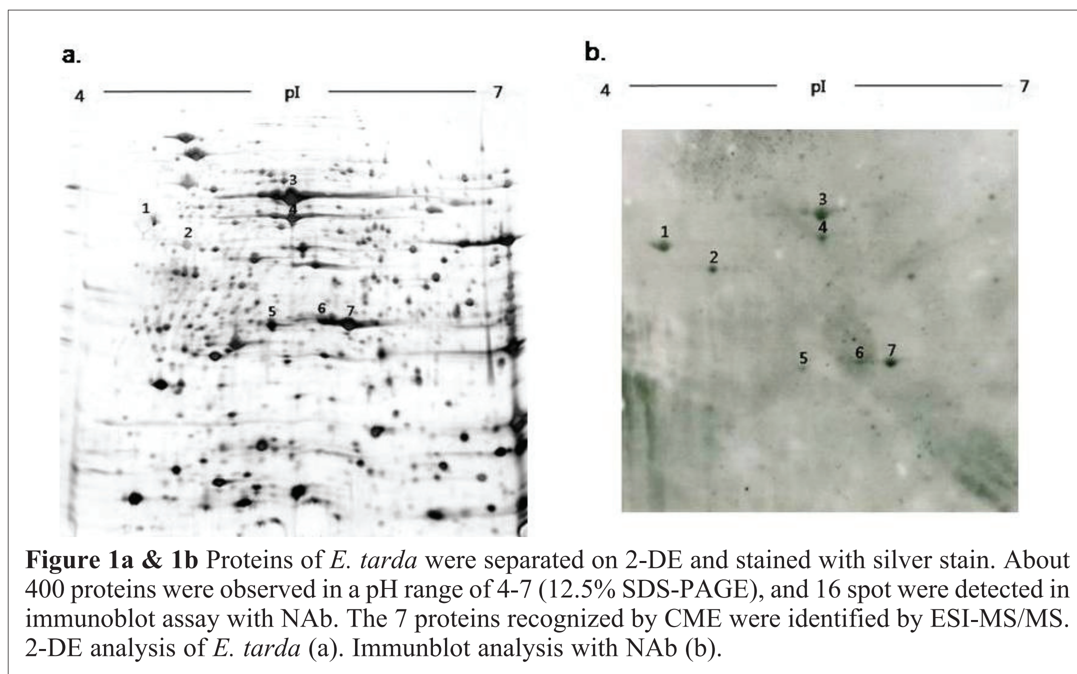

Figure 1a & 1b Proteins of *E. tarda* were separated on 2-DE and stained with silver stain. About 400 proteins were observed in a pH range of 4-7 (12.5% SDS-PAGE), and 16 spot were detected in immunoblot assay with NAb. The 7 proteins recognized by CME were identified by ESI-MS/MS. 2-DE analysis of *E. tarda* (a). Immunblot analysis with NAb (b).

Table 1
Protein identification and peptide homologues

<table>
<tr><th>Spot No.</th><th>Accession no.</th><th>Mascot Score</th><th>Mass/ pI</th><th>Species</th><th>Protein name</th><th>Peptide</th><th>Peptide identity to E. tarda</th></tr>
<tr><td>1</td><td>gi|25989456</td><td>23</td><td>47225/5.3</td><td>Edwardsiella tarda</td><td>outer membrane protein</td><td>R.ANSINTDDIVALGLVYQF.-</td><td></td></tr>
<tr><td>2</td><td>gi|25989456</td><td>59</td><td>47225/5.3</td><td>Edwardsiella tarda</td><td>outer membrane protein</td><td>K.YVDLGATYYFNK.N</td><td></td></tr>
<tr><td>3</td><td>gi|54310177</td><td>71</td><td>45586/4.9</td><td>Photobacterium profundum ss9</td><td>phosphopyruvate hydratase</td><td>K.DVTLAMDCAASEFYDK.E +Carbamidomethyl</td><td>100%</td></tr>
<tr><td>4</td><td>gi|2708662</td><td>71</td><td>6426/4.5</td><td>Edwardsiella ictaluri</td><td>phosphoglycerate kinase</td><td>K.ISYISTGGGAFLEFVEGK.K</td><td>100%</td></tr>
<tr><td>5</td><td>gi|30173331</td><td>47</td><td>22763/5.1</td><td>Enterobacter cloacae</td><td>Ribose-5-phosphate isomerase A</td><td>K.QVDILGNFPLPVEVIPMAR.S</td><td>100%</td></tr>
<tr><td>6</td><td>gi|37520609</td><td>35</td><td>26591/5.4</td><td>Gloeobacter violaceus PCC 7421</td><td>triosephosphate isomerase</td><td>R.LIIAYEPIWAIGTGK.T</td><td>82%</td></tr>
<tr><td>7</td><td>gi|157373037</td><td>38</td><td>26626/5.8</td><td>Serratia proteamaculans 568</td><td>triosephosphate isomerase</td><td>K.GAVIAYEPIWAIGTGK.S</td><td>100%</td></tr>
</table>

DISCUSSION

Under normal conditions, natural antibodies are expressed, even if the individuals are not stimulated by specific antigens acting against non-self substance, such as pathogens, cell debris, nuclear proteins or DNA. This NAb has been believed to IgM in humans (Coutinho *et al.,* 1995). Similarly, in olive flounder, two Igs including IgM and IgM precursor have been observed in the serum (Shin *et al.,* 2007; Bang *et al.,* 1996). In addition, IgM has been observed in the cutaneous mucus of olive flounder, and this has been regarded as a NAb (Palaksha *et al.,* 2008). In this study, the mucosal Nab, IgM, was studied to establish its capability to recognize antigenic proteins of *E. tarda*. The result of immunoblot analysis from 1-DE showed several weak bands between 62 and 25 kDa, which were recognized by CME. As described in a previous study, this result shows the property of NAb of reacting to pathogens by non-specific mechanisms (Coutinho *et al.,* 1995). Therefore, the CME of olive flounder contains NAb and could detect common proteins that had not been previously introduced to the host. To detect the common antigenic proteins, the present study carried out 2-DE and immunoblot assay. Seven proteins from 16 spots which were recognized by CME were identified by ESI-MS/MS. The result of protein identification showed two spots belonging to *E. tarda* and five spots identified from other species. The proteins were identified as belonging to other species due to the limited protein database of *E. tarda* at the time of the experiments. Since then, the whole genomic sequence of *E. tarda* has been published (Wang *et al.,* 2009), and these proteins have been shown to have high sequence identity in BLAST alignment analysis against the published *E. tarda* data. The phosphopyruvate hydratase, phosphoglycerate kinase, and Ribose-5-phosphate isomerase A all showed 100% peptide identity to *E. tarda*. Similarly, triosephosphate isomerase showed 82% and 100% peptide identity to *E. tarda* in *G. violaceus PCC 7421* and *S. proteamaculans 568*, respectively.

In the present study, seven common antigenic proteins of *E. tarda* which can be recognized by NAb, were identified using ESI-MS/MS analysis. Until now, infection mechanisms, attaching sites and penetration are not clearly known. However, abraded skin could be suggested as a penetration site of *E. tarda* (Mohanty and Sahoo, 2007). Therefore, these common proteins might be important epitopes of *E. tarda* which are recognized by NAb, and advances the understanding of the pathogen invasion mechanism on the skin of olive flounder; especially, the role of NAb to recognize the specific epitope against *E. tarda* infection. However, the present study only showed *E. tarda* common antigens; thus, information of common antigenic proteins from other infectious species might be required to clearly understand the character of NAb in the cutaneous mucus of olive flounder.

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Immunoproteomic analysis of extracellular products (ECPs) obtained from three etiological isolates of streptococcosis (*Streptococcus iniae, Streptococcus parauberis* Dongbo and *Streptococcus parauberis* Namhae) of olive flounder (*Paralichthys Olivaceous*) in Korea

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ABSTRACT

In the pathogenesis of *Streptococcus sp*. in mammals, extracellular proteins (ECPs) have been a well critical factor in immunological invasion and evasion. Yet, in fish, there have only been a few reports on the immunological role of ECPs in streptococcosis. Recently, ECPs have been evaluated as an effective immunogenic source against *Streptococcus iniae* infection in olive flounder. Here, we characterize ECPs associated etiological bacteriacausing streptococcosis in olive flounder: *Streptococcus iniae, Streptococcus parauberis* Dongbo, and *Streptococcus parauberis* Namhae. Immunoproteomic analysis in which ECPs from the respective bacteria reacted with olive flounder antisera against homologous/heterologous bacteria or different strains was carried out. In 2-DE immunoblot profile ECPs, antigenic spots were identified as Secretion protein Bug4, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, Enolase, 60 kDa chaperonin, phosphoglycerate kinase, DNA-directed RNA polymerase alpha chain, and elongation factor Tu. This study might provide useful data to understand ECPs protein composition,

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it's role in pathogenesis, and immunogenicity by using proteomics techniques such as mass spectrometry (MS and MS/MS) and peptide mass fingerprinting (PMF). Further studies will evaluate the efficacy of adding ECPs to *Streptococcus sp.* vaccine.

Key words: Olive flounder; *Streptococcus iniae*; *Streptococcus parauberis*; extracellular proteins; vaccine.

INTRODUCTION

Olive flounder (*Paralichthys olivaceus*) is a commercially important marine species in Korea, China and Japan. Currently, the production of this species yielded 43 metric tons and earned USD 200 M in 2008 (Statistics Korea, www.kostat.go.kr). However, the intensive culture of this species has resulted to severe economic losses by various bacterial diseases, especially streptococcosis (Shin *et al*., 2007a). The major etiological agents of streptococcosis include *Streptococcus iniae* and *S. parauberis* (Nho *et al*., 2009). Hence, a general vaccine against *Streptococcus sp.* should be developed to effectively prevent streptococcosis induced by the co-infection of a variety of bacteria (Toranzo *et al*., 2005). However several attempts using a homologous-inactivated/ killed whole cell vaccine (bacterin) still focused on antigenic variation, since, along with several vaccination conditions (Romalde *et al*., 2005; Toranzo *et al*., 2005), the efficacy could also vary with the strains and/or isolates (Bachrach *et al*., 2001; Eldar *et al*., 1997; Klesius *et al*., 2000). In addition**,** little was known about cross-protection of respective vaccine against such pathogens. Previous studies have shown that streptococcal ECPs could be the key to improve cross-protection; for example, the modified *S. agalactiae* bacterin (containing ECPs) provided protection against homologous and heterologous challenge without booster in tilapia (Evans *et al*., 2004). It is likely that ECPs could be important materials to provoke cross-protection against various streptococcocal agents. Immunoproteomics has been employed in various studies to effectively investigate not only immune response between host and pathogen, but also antigenic proteins of importance for vaccine developments (Ni *et al*., 2010; Shin *et al*., 2007a; Vytvytska *et al*., 2002). In this study, we applied an immunoproteomic approach (the combination of 2-dimensional electrophoresis (2-DE) and immunoblotting with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)) to explore the common antigenic proteins from ECPs of etiological agents, two strains isolated from different regions including *S. iniae* Jeju45 (Shin *et al*., 2007c), *S. parauberis* Dongbo (Korea Jeju province) (Nho *et al*., 2009) and *S. parauberis* Namhae (Korea Gyeongnam province) (Korea isolate). The information on secreted antigenic proteins relevant to cross-reactivity might be helpful in understanding the role of ECPs in pathogenicity and antigenicity.

MATERIALS AND METHODS

Bacteria

The *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae used in this study were isolated from spleen of diseased flounder in 2000, 2006 and 2009 (Shin *et al*., 2007c; Nho *et al*., 2009). These isolates were incubated in Tryptic Soya Broth (TSB, OXOID) and Tryptic Soya Broth (TSB, OXOID) at 25°C for 24h.

Production of olive flounder antiserum

Olive flounder specific anti- *S. iniae, S. parauberis* Dongbo and *S. paraubeirs* Namhae sera were produced as described by Kang *et al.* (2006). Olive flounder with an average body weight of 150g and an average length of 15 cm were purchased from a commercial fish farm located in Namhae county on the southern coast of Korea. Fish were divided into 4 groups of 5 individuals each and were maintained in 200 L FRP tanks at 25°C. The fishes were intraperitoneally injected with 0.1 ml (approximately 10^7 CFU ml^{-1}) of *S. iniae, S. parauberis* Dongbo and *S. paraubeirs* Namhae. Live bacteria were used to ensure the immunogenicity of the bacteria is maintained. Fishes from one tank was maintained as negative control. After 2 weeks, blood was collected from the recovered flounder and the upper supernatant was separated by centrifuge at 3,500 rpm for 15 min at 4°C.

ECPs precipitation

Extracellular precipitation was performed as described by Evans *et al.* (2004) and modified. For preparation of bacterial ECPs, *S. iniae* Jeju-45, *S. parauberis* Dongbo *and S. parauberis* Namhae isolates were cultured on TSB for 24 h at 25°C. The supernatants were obtained by centrifugation at 8,000×*g* for 30 min and the cell-free fluid was sterilized using 0.45 μm disposable filter. The ECPs was dehydrated with a freeze dryer (Heto MAXI dry lyo, Allerod, Denmark) and stored at -70°C until use. The ECPs powder was diluted by sterilized PBS, the protein concentrations were determined by Bradford assay using bovine serum albumin as a standard.

Isoelectric focusing (IEF) and SDS-PAGE

IEF was performed using the IPGphorTM system (Amersham Bioscience, Uppsala, Sweden) with IPG strips (Immobiline DryStripTM, pH 4–7, 13 cm; Amersham Bioscience), according to the previously reported method (Shin *et al*., 2006). The protein loading volume for ECPs extract was adjusted to 120 μg ml^{-1} with rehydration buffer (9M urea, 2% CHAPS, 0.4% DTT, 0.5% IPG-buffer, and 0.002% bromophenol blue. Prepared sample was loaded onto the IPG strips and focused at 86.1 kV h at 20 °C using an automated system as follows: rehydration for 12 h (6 h at 30V followed by 6 h at 60 V), and focusing for 17 h (2 h at 200V, 1 h at 500V, 1 h 1000V, 1 h at 2000V, 2 h at 4000V and 10 h at 8000 V). After IEF, the IPG strip was equilibrated with 10 mg ml^{-1} of DTT in equilibration buffer (6M urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, and 50mM Tris–HCl, pH 8.8) for 15 min, and then with 25 mg ml^{-1} of iodoacetamide in the equilibration buffer for another 15 min. Equilibrated IPG strip was placed onto 12.5% SDS-polyacrylamide gels

(18 cm×16 cm×0.1 cm), sealed with 0.5%, w/v low melting agarose (Sigma), then electrophoresis at 10mA gel^{-1} for 15 min followed by application of 20mA gel^{-1} until the dye reached the bottom of the gel, and silver-stained.

2-DE immunoblot assay

For 2-DE immunoblot assays, each ECPs contained in spots of 2DE gels was transferred to a PVDF membrane (Millipore, USA). The membranes were blocked with 5 % skim milk in PBS-T for 1 h at room temperature (RT), washed three times with PBS-T, incubated with flounder specific anti- *S. iniae* Jeju-45 sera, anti-*S. parauberis* Dongbo sera and anti-*S. parauberis* Namhae sera for 2 h at RT, respectively and washed three times with PBS-T for 15 min. Second antibody, olive flounder IgM monoclonal antibody (Shin *et al*., 2007b) was incubated for 2 h at RT and washed three times with PBS-T for 15 min. Antigenic spots from ECPs were visualized by incubation with goat anti-mouse-HRP (Jackson, USA; 1:4000) for 1 h at RT. After washing with PBS-T, the membrane was developed using an Enhanced Chemiluminescent (ECL) kit (Amersham Biosciences) then exposed to X-ray film for visualization of the antigenic proteins. Images of stained gels and X-ray films were digitalized with an EPSON perfection V700 photo image scanner (SEIKO EPSON CORP, USA) and the acquired images were analyzed using Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK).

MALDI-TOF MS

Protein spots were identified by Peptide Mass Fingerprinting (PMF) using Mass-Assisted Laser Desoption/Ionization Mass Spectrometry (MALDI-TOF MS) as described by Shin *et al*. (2006). In brief, protein spots of interest were excised from the silver-stained gels and subjected to in-gel digestion with 12.5 ng/ml porcine trypsin (Promega, Madison, WI, USA) at 37°C overnight (approximately 16 h). The supernatant was recovered and extracted twice with an equal volume of 5% formic acid and acetonitrile, and the extracts were pooled and dried in a vacuum centrifuge. Dried tryptic peptides were redissolved in 1 ml of sample solution (93:5:2, v/v ratio of DW, acetonitrile and TFA), and targeting on MALDI plates was performed using the solution-phage nitrocellulose method (Lee *et al*., 2003). Alpha-cyano-4-hydroxycinamic acid (40 mg/ml) and nitrocellulose (20 mg/ml) were prepared separately in acetone and mixed with isopropanol in a ratio of 2:1:1 (v/v). The internal standards, des-Arg-Bradykinin (monoisotopic mass: 904.4681) and angiotensin I (1296.6853) (Sigma–Aldrich, St. Louis, MO, USA) were added to the mixture to generate the matrix solution. The1 µl matrix solution was spotted onto target circles on the MALDI plate and dried. The dried samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The proteins were identified by comparing the obtained mass spectra to the National Center for Biotechnology Information (NCBI) and SwissProt protein sequence databases using the Prospector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) website.

RESULTS AND DISCUSSION

Proteins were separated based on their isoelectric points using 2-DE. On 2-DE, approximately 87, 98 and 95 spots were observed for *S. iniae* Jeju45*, S. parauberis* Dongbo and *S. parauberis* Namhae ECPs, respectively (Fig. 1A, Fig. 2E and Fig. 3I). In the 2-DE immunoblot assay, serum raised against *S. iniae* Jeju45*, S. parauberis* Dongbo and *S. parauberis* Namhae were used due to its ability to recognize the ECPs containing immune reactive spots three bacteria isolates (Fig. 1, 2 and 3). All the immunoreactive spots were also observed in respective duplicated 2-DE gel.

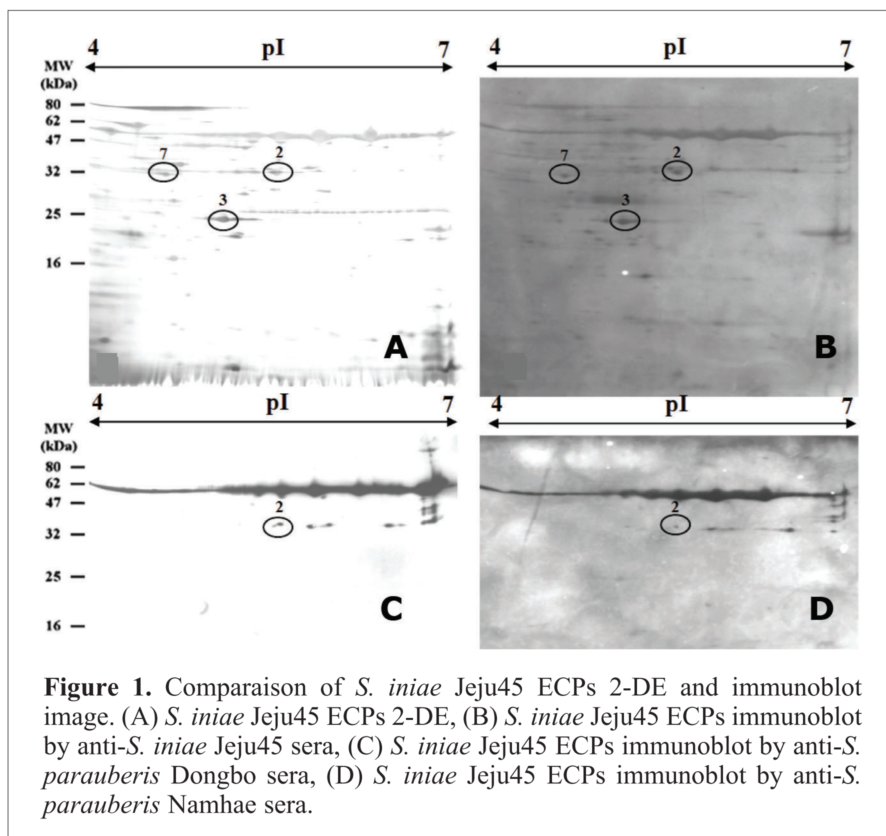

Figure 1. Comparaison of *S. iniae* Jeju45 ECPs 2-DE and immunoblot image. (A) *S. iniae* Jeju45 ECPs 2-DE, (B) *S. iniae* Jeju45 ECPs immunoblot by anti-*S. iniae* Jeju45 sera, (C) *S. iniae* Jeju45 ECPs immunoblot by anti-*S. parauberis* Dongbo sera, (D) *S. iniae* Jeju45 ECPs immunoblot by anti-*S. parauberis* Namhae sera.

Analysis of cross reactivity using *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae ECPs and its immunoproteomics contained antigenic proteins such as secretion protein Bug4, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Fructose-bisphosphate aldolase, enlase, 60 kDa chaperonin, phasphoglycerate kinase, DNA-directed RNA polymerase α chain and elongation factor-TU. GAPDH and Fructose-bisphosphate aldolase were commonly elicited against homologous/ heterologous isolates and different genera. These are potentially important antigenic proteins of *Streptococcus* sp. (Table 1).

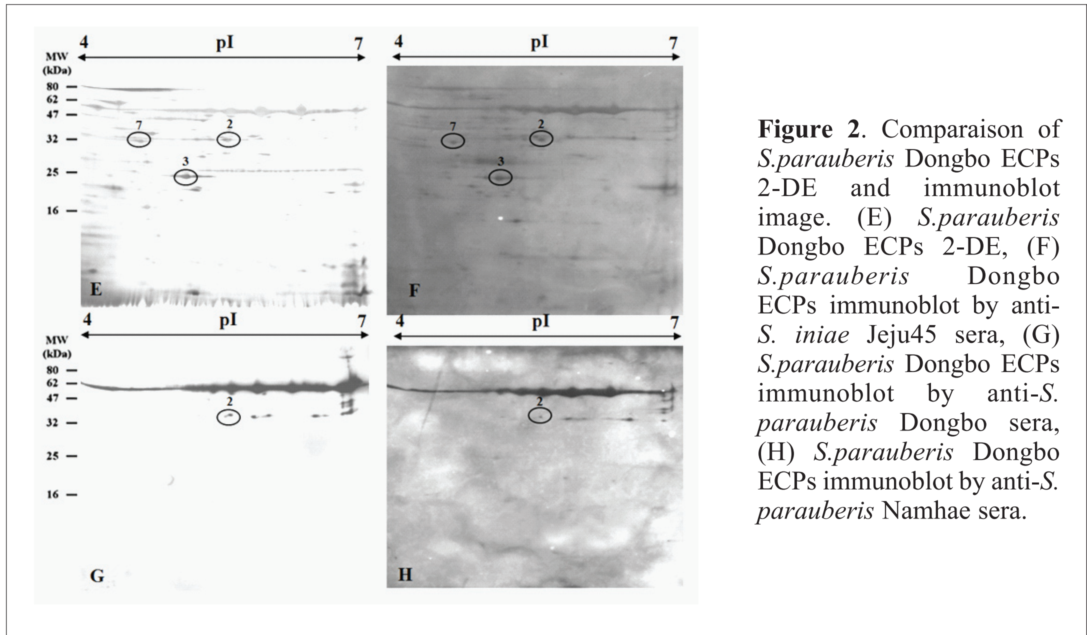

Figure 2. Comparaison of *S.parauberis* Dongbo ECPs 2-DE and immunoblot image. (E) *S.parauberis* Dongbo ECPs 2-DE, (F) *S.parauberis* Dongbo ECPs immunoblot by anti-*S. iniae* Jeju45 sera, (G) *S.parauberis* Dongbo ECPs immunoblot by anti-*S. parauberis* Dongbo sera, (H) *S.parauberis* Dongbo ECPs immunoblot by anti-*S. parauberis* Namhae sera.

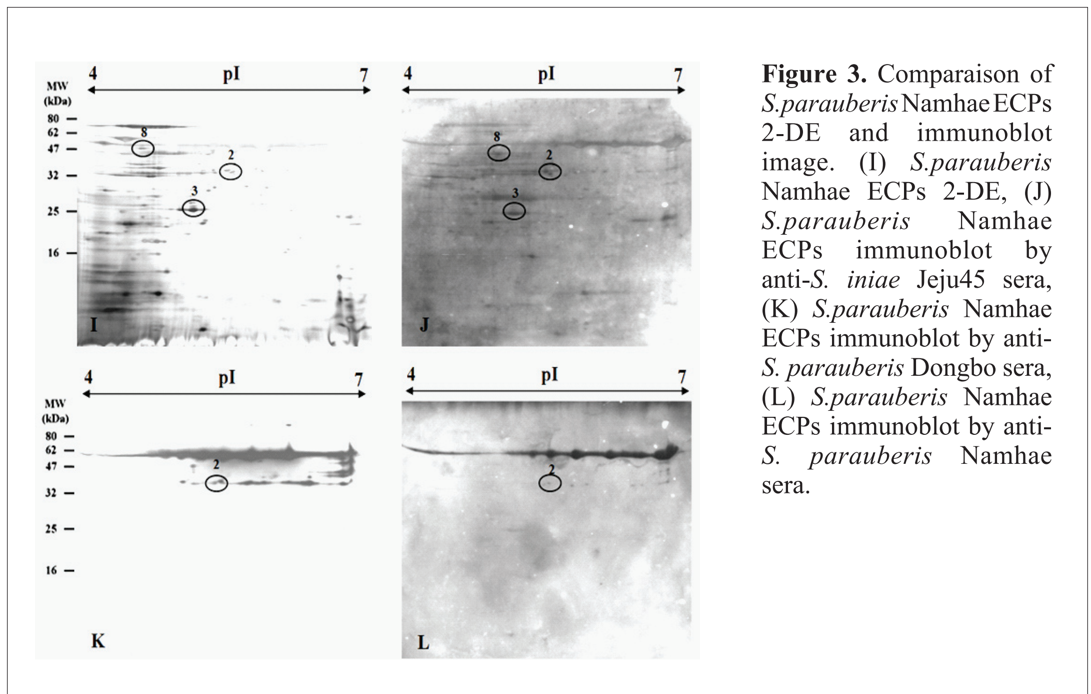

Figure 3. Comparaison of *S.parauberis* Namhae ECPs 2-DE and immunoblot image. (I) *S.parauberis* Namhae ECPs 2-DE, (J) *S.parauberis* Namhae ECPs immunoblot by anti-*S. iniae* Jeju45 sera, (K) *S.parauberis* Namhae ECPs immunoblot by anti-*S. parauberis* Dongbo sera, (L) *S.parauberis* Namhae ECPs immunoblot by anti-*S. parauberis* Namhae sera.

Table 1
The list of identified antigenic proteins of *S. iniae* Jeju45, *S.parauberis* Dongbo and *S.parauberis* Namhae ECPs 2-DE and immunoblot.

Spot No.	Figure image / Antigenic proteins	S. iniae Jeju45 ECPs 2-DE and immunoblot image				S. parauberis Dongbo ECPs 2-DE and immunoblot image				S. parauberis Namhae ECPs 2-DE and immunoblot image			
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
1.	Secretion protein Bug4	O	O	O	O	X	X	X	X	X	X	X	X
2.	glyceraldehyde-3-phosphate dehydrogenase	O	O	O	X	O	O	O	O	O	O	O	O
3.	fructose-bisphosphate aldolase	O	O	X	X	O	O	X	X	O	O	X	X
4.	Enolase	O	O	X	X	X	X	X	X	X	X	X	X
5.	60 kDa chaperonin	O	O	X	X	X	X	X	X	X	X	X	X
6.	phosphoglycerate kinase	O	O	X	X	X	X	X	X	X	X	X	X
7.	DNA-directed RNA polymerase alpha chain	X	X	X	X	O	O	X	X	X	X	X	X
8.	elongation factor Tu (EF-TU)	X	X	X	X	X	X	X	X	O	O	X	X

GAPDH and enolase known as located on the cell wall in streptococcus species, and transported to the cell surface without recognizable signal sequences. In addition GAPDH and enolase was determined to serve as the streptocococcal plasmin receptor involved in bacterial adhesion and signal transduction to host cells (Bisno *et al*., 2003; Cunningham, 2000). And 60 kDa chaperonin and elongation factor-Tu have a dual function and serves as both a ribosomal structural protein that binds rRNA and a translational repressor for overcome to varied environment (Len *et al*., 2004). Phosphoglycerate kinase provided a regulatory link between glycolytic activity and signal transduction regulation involving biofilm formation for protective antigen (Iwami and Yamada., 1985) Others also contributed the pathogen survival or/and virulence acquisition such as DNA replication, enzyme synthesis, biosynthesis of metabolic cycle and sugar utilization.

Based on these result, it could be suggested that *S. iniae* Jeju45*, S. parauberis* Dongbo and *S. parauberis* Namhae might share structural constituents of secreted proteins of three bacterial isolates as common immunogens, which could provide cross-protection against etiological agents.

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Antimicrobial peptides from the black tiger shrimp *Penaeus monodon* – a review

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ABSTRACT

Black tiger shrimp *Penaeus monodon* is one of the major shrimp species being cultured in Asian countries. Disease outbreaks have resulted in a decline of *P. monodon* cultivation and severe losses in the shrimp production. To combat harmful microorganisms, shrimp produce diverse classes of antimicrobial peptides (AMPs) as part of their immune defense. In *P. monodon*, several cDNA sequences of AMPs were identified by means of expressed sequence tag (EST) approach. These DNA sequences encode different putative AMPs, such as crustins, penaeidins, anti-lipopolysaccharide factors (ALFs) and lysozymes. In this review, we present the recent information on sequence diversity, expression and antimicrobial properties of *P. monodon* AMPs. The information indicates the importance of these AMPs as the effective host defense molecules against the invasion of pathogenic microorganisms.

Keywords: *Penaeus monodon*, shrimp, antimicrobial peptides, innate immunity, expressed sequence tag

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INTRODUCTION

Antimicrobial peptides (AMPs), ubiquitously found in all living organisms, are important immune effectors with an ability to neutralize and/or kill invading microorganisms (Brown and Hancock, 2006). AMPs share common features of small size (about 15 to 100 amino acids) with amphipathic properties but they differ in the primary sequences, secondary structures and spectra of activities. Lacking the adaptive immunity, shrimp rely primarily on immune molecules including AMPs to combat the varieties of microorganisms. The first AMP family discovered in shrimp was penaeidin of the Pacific white shrimp, *Litopenaeus vannamei* (Destoumieux *et al.*, 1997) which displayed antimicrobial activity against Gram-positive bacteria and fungi. Thereafter, other shrimp AMP families: crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes (Amparyup *et al.*, 2008a; Amparyup *et al.*, 2008b; Bartlett *et al.*, 2002; Hikima *et al.*, 2003; Sotelo-Mundo *et al.*, 2003; Supungul *et al.*, 2004), had been reported in various shrimp species. These shrimp AMPs have been characterized for their expression and antimicrobial activities. In the black tiger shrimp *Penaeus monodon*, Supungul *et al*. (2002; 2004) reported the discovery of cationic AMPs from the hemocyte cDNA libraries. The most recent data was reported by Tassanakajon *et al*. (2006) describing the identification of putative antimicrobial sequences from the *Penaeus monodon* EST database http://pmonodon.biotec.or.th. In this paper, we summarized a recent information on *P. monodon* AMPs concerning the sequence variations, gene expression in various tissues and in response to pathogen infections and antimicrobial properties.

SEQUENCE DIVERSITY

AMPs found in *P. monodon* were discovered mainly by means of expressed sequence tag (EST) analysis. A total of 889 ESTs encoding putative AMPs were identified in the *Penaeus monodon* EST database of 40,001 EST sequences. These AMP sequences represent four major AMPs: penaeidins, crustins including single WAP domain (SWD) proteins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Of these AMPs, crustins were the most abundant followed by penaeidins, ALFs and lysozymes, respectively (Table 1).

Crustins are cationic cysteine-rich antibacterial peptides consisting of a single whey acidic protein (WAP) domain (Smith *et al*., 2008). The WAP domain located at the C-terminus of the molecule contains 50 amino acid residues with eight cysteine residues at defined positions (Ranganathan *et al*., 1999). The first crustin molecule, an 11.5 kDa antimicrobial protein namely 'carcinin', was isolated from hemocytes of the crab *Carcinus maenas* (Smith and Chisholm, 2001). The putative crustin genes of *P. monodon* (crustin*Pm*s) have been identified from the hemocyte and the gill-epipodite cDNA libraries (Supungul *et al.*, 2004; Tassanakajon *et al*., 2008; Vatanavicharn *et al.*, 2009). In comparison with other *P. monodon* AMPs, the crustin family exhibits the highest sequence variation both in length and their primary sequences. According to the classification of crustins proposed by Smith *et al.* (2008), *P. monodon* crustins belong to Type II and III crustins. Type II crustin constitutes the majority of *P. monodon* crustin sequences consisting of seven isoforms (crustin*Pm*s1-

Table 1
Types, number of sequences, diversity and antimicrobial activities of the *Penaeus monodon* AMPs from the EST database (http://pmonodon.biotec.or.th)

Types	No. of sequences	No. of isoforms	Major isoform	Antimicrobial activities	References
Crustins					
Type II	275	7	Crustin*Pm* 1	Gram +	Supungul *et al*., 2008
			Crustin*Pm* 5	Gram +	Vatanavicharn *et al*., 2009
			Crustin-like *Pm*	Gram +	Amparyup *et al*., 2008a
				Gram +, Gram-	
Type III (SWD)	50	3	SWD*Pm*2	Gram+, anti-subtilisin	Amparyup *et al*., 2008b
ALFs	208	6	ALF*Pm*3	Gram +, Gram-, fungi	Somboonwiwat *et al*., 2005
				Anti-virus	
					Thartada *et al*., 2009
			ALF*Pm*2	Gram +, Gram-	Tharntada *et al*., 2008
Penaeidins	284	2	PEN3	ND*	Tassanakajon *et al*., 2008
			PEN 5	ND*	Tassanakajon *et al*., 2008
Lysozymes	72	2	C-type	Gram+, Gram-	Supungul *et al*.,
			I-type	Gram+, Gram-	2010

*ND is no data available.

6 and crustin-like*Pm*) whereas Type III crustin or single WAP domain containing protein (SWD) comprises three isoforms of SWD (SWD*Pm*s 1-3).

Type II crustins contains a signal peptide followed by a long glycine-rich domain and a cysteine-rich domain (four cysteine residues) at the N-terminal region, and a WAP domain (eight cysteine residues) at the C-terminal region. Based on the alignment of amino acid sequences, all Type II crustin*Pm*s possess 12 cysteine residues, eight of which participate in a four disulfide core (4DSC) or WAP domain. High sequence diversity both in length and primary sequence of different isoforms of crustin*Pm*s was clearly observed (Tassanakajon *et al*., 2008). Recent data showed that crustin*Pm*s were encoded by different genes and had quite different promoter and regulatory sequences. Genome organization study of crustin-like*Pm* gene (716 bp) reveals that it consists of two exons (38 and 487 bp) and one intron (191 bp) (Amparyup *et al*., 2008b). The 5'UTR sequence contains a putative TATA box and several potential *cis*-acting elements, e.g. putative binding sites for GATA binding factor, STAT5, NF-kappaB, AP-1 and C/EBP-b. Crustin*Pm*5 gene (1,394 bp) contains four exons of 31, 75, 242 and 194 bp, respectively, interrupted by three introns of 606, 148 and 116 bp. Instead of the putative GATA and NF-kappaB sites, interestingly, the upstream sequence of

the crustin*Pm*5 gene contains a complete heat shock regulatory element (HSE) suggesting that it is the heat-inducible gene (Vatanavicharn *et al*., 2009).

The less well-characterized *P. monodon* Type III crustin or SWD*Pm* is composed of 3 subgroups (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) (Amparyup *et al*., 2008a). The Type III crustin does not contain the glycine-rich and the cysteine-rich regions but instead has a proline-arginine rich region between the signal sequence and the WAP domain. Analysis of the exon-intron organization of Type III crustin genes from *P. monodon* SWD (Chen *et al*., 2006) and SWD*Pm*2 (Amparyup *et al*., 2008a) reveals that they both contain three exons and two introns. The differences between these two SWD proteins are the size of the first intron and the microsatellite sequences in the second intron. At the 5' upstream sequences, putative transcription factor binding sites, such as cap, hsf, GATA-1, SRY, Tst-1, HNF-3b, CF2-II, Oct-1, AP-1 and USF, are found but not the putative TATA box (Chen *et al*., 2006).

As mentioned before, the penaeidin family is the first AMP identified and characterized in penaeid shrimp (Destoumieux *et al*., 1997). Penaeidins are cationic peptides with a molecular mass of 5.5 to 6.6 kDa. They have two domains: one is an N-terminal proline-rich domain and the other is a C-terminal cysteine-rich domain containing six cysteine residues. A collection of penaeidin sequences has been reported in PenBase www.penbase.immunaqua.com established in 2006 (Gueguen *et al*., 2006). Penaeidins have variation in the primary sequences and can be grouped into four classes (PEN2, PEN3, PEN4 and PEN5). Amino acid sequence characteristics and conserved key residues proposed as penaeidin signature of PEN2, PEN3 and PEN4 were previously described in PenBase whilst those of PEN5 were proposed by Kang *et al.* (2007). However, recent alignment of known penaeidin sequences revealed that there were variations in a few key amino acid residues in the proposed penaeidin signatures (Tassanakajon *et al*., 2011).

In *P. monodon*, only PEN3 and PEN5 were identified and PEN3 is the most abundant class (Tassanakajon *et al*., 2006). The members of PEN3 show the differences in the number and sequences of amino acids across the shrimp species, but within the species the members of PEN3 from *P. monodon* have the same amino acid residue in length (74 aa). Generally, the variants arise from the amino acid variations in the proline-rich domain. The *P. monodon* PEN5 exhibits 72% sequence identity to those from *Fenneropenaeus chinensis* (Woramongkolchai *et al*., 2011). Unlike the intronless PEN3 gene from *L. vannamei* (O'Leary and Gross, 2006), genomic structure analysis of PEN3 from *P. monodon* by Chiou *et al.* (2007) indicated that it contains an intron of 680 bp. Also, the PEN5 from *P. monodon* contains an intron of 620 bp (Woramongkolchai *et al*., 2011).

ALFs are the antimicrobial peptides firstly isolated from the hemocytes of the horseshoe crabs, *Tachypleus tridentatus* and *Limulus polyphemus* (Tanaka *et al*., 1982; Muta *et al*., 1987). ALF genes have, then, been identified from several species of crustacean including shrimp (Supungul *et al*., 2002; Liu *et al*., 2006; Nagoshi *et al*., 2006; Rosa *et al*., 2008; Beale *et al*., 2008; Imjongjirak *et al*., 2007). The *P. monodon* ALFs (ALF*Pms*) constitute at least five different isoforms (Supungul *et al.,* 2002) which are grouped into groups A

(ALF*Pm*1 and 2) and B (ALF*Pm*3-5), derived from different genomic loci, according to nucleotide sequence analysis (Tharntada *et al*., 2008). Group A ALF*Pm* contains three exons interrupted by two introns, but group B is composed of four exons interrupted by three introns. The different isoforms within the group derived from the alternative RNA splicing phenomenon. However, ALF*Pm*s share common features of high hydrophobicity at the N-terminal region and the conserved disulfide loop containing positively charge cluster, described as the putative LPS-binding domain (Hoess *et al.*, 1993). ALF*Pm* genes contain an open reading frame encoding a protein of 98 - 123 amino acid residues.

The cis-regulatory elements involving in immune response and/or regulating the expression of antimicrobial peptides, such as NF-kappaB, GATA, AP-1, GAAA and Oct-1 motifs are found in the genomic sequences at the 5' upstream region of both ALF*Pm* groups A and B. However, the AP-1 and NF-kappaB binding sites are only predicted in the ALF group A genes. It should be noted that the presence of AP-1 (Karin *et al.,* 1997; Douglas *et al.,* 2003) together with the GAAA motifs (Au *et al.,* 1993) infers that the ALF*Pm* group A genes expressed in response to viral infection.

Lysozyme is an important antibacterial protein that hydrolyzes bacterial cell wall leading to cell lysis. Lysozyme, normally found in both eukaryotes and prokaryotes, can be divided into six types: plant lysozyme, chicken-type lysozyme (c-type), goose-type lysozyme (g-type), invertebrate lysozyme (i-type), bacteria lysozyme and T4 phage lysozyme (phage-type). In *P. monodon*, a total of 72 EST sequences encoding lysozymes have been identified. Of these, only two types of lysozyme, c-type and i-type have been identified (Table 1) (Tassanakajon *et al*., 2008; Supungul *et al*., 2010). The information on sequence diversity and the genomic structures of *P. monodon* lysozymes are being analyzed.

GENE EXPRESSION PATTERNS

Gene expression of *P. mondon* AMPs in various shrimp tissues, during the developmental stages and in response to pathogen infection, has been extensively investigated and is discussed herein.

Generally, AMP genes are highly expressed in shrimp hemocytes but lower expression is observed in other tissues such as intestine, heart, gill, and lymphoid organ (Tassanakajon *et al*., unpublished data). However, some isoforms of *P. monodon* AMPs are specifically expressed in particular tissues which imply the specific functions or responses of those isoforms.

The crustin-like*Pm* gene is highly expressed in *P. monodon* hemocytes and weakly expressed in gills and intestines. On the other hand, crustin*Pm*5 gene is somehow expressed only in epipodites and eyestalks but not in hemocytes. In addition, crustin*Pm*5 gene expression is found to be up-regulated in response to heat and hyperosmotic salinity stresses (Vatanavicharn *et al*., 2009). These data perhaps indicate the function of crustins other than antimicrobial activity as effectors or mediators in physiological stress responses.

SWD*Pm*s are all mainly expressed in hemoctyes. SWD*Pm*1 and SWD*Pm*3 mRNA expressions are hemocyte specific whilst SWD*Pm*2 transcripts are abundant in hemoctyes but lower amounts are also found in gills and hepatopancreas. There is no SWD*Pm* transcripts observed in the lymphoid organ, intestine and heart.

So far, all ALF*Pm* isoforms are shown to be specifically expressed in hemocytes with variation in the expression level (Somboonwiwat *et al*., 2006; Somboonwiwat *et al.*, 2008; Tharntada *et al*., 2008). Hemocyte is also the main site of penaeidin gene expression as reported in *L. vannamei* (Destoumieux *et al*., 2000). Expression analysis of *F. chinensis* PEN5 also reveals that it is constitutively expressed in hemocytes, heart, gills, intestine and ovary (Kang *et al*., 2007). In our study, *P. monodon* PEN3 is expressed in all tissues tested: hemocyte, lymphoid organ, gill, hepatopancreas, heart and intestine, with the highest level in hemocyte (Tassanakajon *et al*., unpublished data).

Shrimp is exposed to a variety of microorganisms in the environment throughout their developmental stages. During *P. monodon* development, it is found that all AMPs are expressed at the very early developmental stages starting from the nauplii stage to adult (Tassanakajon *et al*., unpublished data) (Fig. 1). The results suggest that the presence of AMPs is vital to the shrimp throughout their development.

Under pathogenic infections, *P. monodon* AMP genes have different expression patterns. Vagas-Albores *et al.* (2004) reported that the crustin isoform I mRNA of *L. vannamei* was down-regulated at 12 to 24 h post-*Vibrio alginolyticus* injection but the isoform P transcript was constantly expressed. On the contrary, the crustin-like*Pm* was significantly

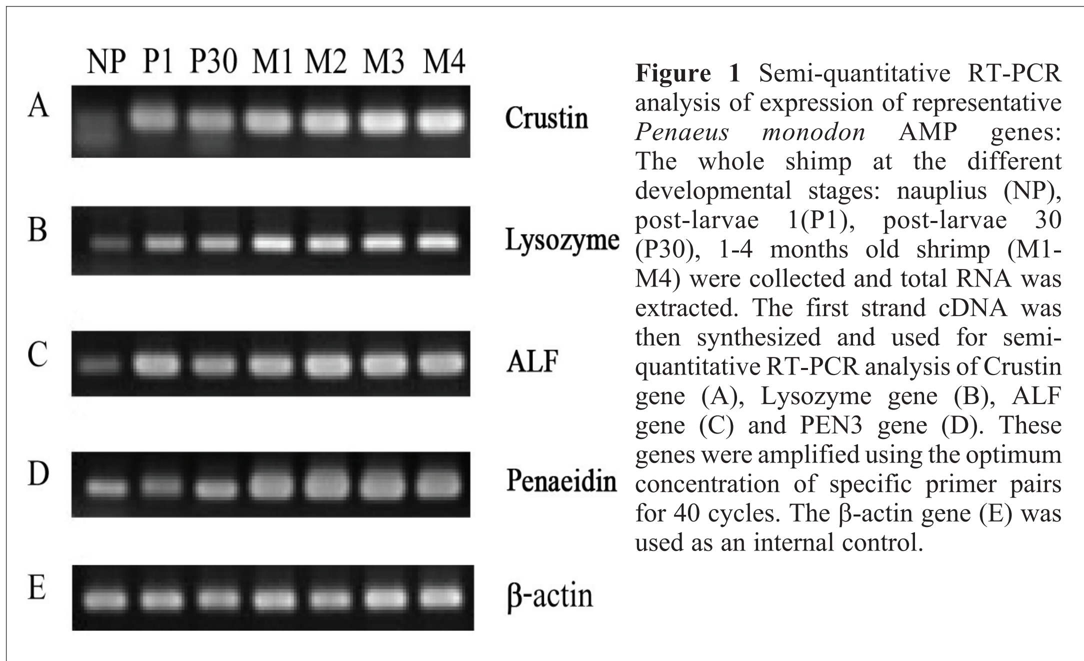

Figure 1 Semi-quantitative RT-PCR analysis of expression of representative *Penaeus monodon* AMP genes: The whole shimp at the different developmental stages: nauplius (NP), post-larvae 1(P1), post-larvae 30 (P30), 1-4 months old shrimp (M1-M4) were collected and total RNA was extracted. The first strand cDNA was then synthesized and used for semi-quantitative RT-PCR analysis of Crustin gene (A), Lysozyme gene (B), ALF gene (C) and PEN3 gene (D). These genes were amplified using the optimum concentration of specific primer pairs for 40 cycles. The β-actin gene (E) was used as an internal control.

up-regulated at 24 h post *V. harveyi* challenge (Amparyup *et al*., 2008b). SWD*Pm*1 mRNA level was decreased sharply at 6 h after *Staphylococcus aureus* injection where no change in expression level of SWD*Pm*2 and SWD*Pm*3 was evident (Amparyup *et al*., 2008a). *V. harveyi* infection also causes rapid up-regulation of ALF*Pm*3 gene expression in hemocytes (Somboonwiwat *et al*., 2006; Somboonwiwat *et al*., 2008).

Upon white spot syndrome (WSSV) infection, the expression of AMPs from *P. monodon* such as ALF*Pm*2, ALF*Pm*3, Crustin*Pm*1, Crustin-like*Pm*, SWD*Pm*1-3, PEN3 and PEN5, was investigated by a semi-quantitative RT-PCR (Amparyup *et al*., 2008a; Tassanakajon *et al*., unpublished data). The data revealed that the expression of ALF*Pm*3, ALF*Pm*6, SWD*Pm*1, SWD*Pm*2 and PEN5 considerably was increased at different time points, whereas those of others were not dramatically changed. The results suggested the important role of *P. monodon* AMPs in antiviral immune responses.

ANTIMICROBIAL ACTIVITY

In order to study the biological activity of AMPs from *P. monodon*, the recombinant proteins of the major isoforms of each AMP family including ALF*Pm*2, ALF*Pm*3, crustin*Pm*1, crustin-like*Pm*, crustin*Pm*5, SWD*Pm*2, c-type lysozyme and i-type lysozyme, wwere produced and tested for their activities (Somboonwiwat *et al*., 2005; Supungul *et al*., 2008; Amparyup *et al*., 2008a, Amparyup *et al*., 2008b; Vatanavicharn *et al*., 2009). Antimicrobial assays demonstrated that almost all recombinant AMPs from *P. monodon* showed narrow spectrum of activity against specific target bacteria except for the recombinant ALF*Pm*3 protein (rALF*Pm*3) that showed wide range and strong activity against Gram-positive and Gram-negative bacteria and fungi. The recombinant proteins of crustin*Pm*1, crustin*Pm*5 and SWD*Pm*2 exhibit only anti-Gram-positive bacteria whilst the recombinant crus-like*Pm* has strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. The preliminary data on the activity of *P. monodon* lysozyme indicated that both c-type and i-type lysozymes were active against Gram-negative bacterium *V. harveyi*. Moreover, the recombinant c-type lysozyme also inhibited the growth of a Gram-positive bacterium *Micrococcus luteus*. Inhibition study had revealed that the antimicrobial activities of ALF*Pm*3 and crustin*Pm*1 were a result of bactericidal effect. In addition, SWD*Pm*2 also has antiproteinase activity against subtilisin A. It should be also emphasized herein that not all but several AMPs are active against *V. harveyi*, a major pathogenic bacteria in shrimp aquaculture.

In vivo neutralization activity and protective effects of rALF*Pm*3 were recently reported in *P. monodon* challenged with *V. harveyi* (Ponprateep *et al*., 2009). It was found that *V. harveyi* at a lethal dose was completely neutralized by pre-incubation of live bacteria with rALF*Pm*3 at the lowest concentration of 6.25 µM resulting in 100% shrimp survival after systemic challenge. The rALF*Pm*3 injection into the shrimp followed by *V. harveyi* challenge clearly showed the reduction of the cumulative mortality of shrimp suggesting its potential prophylactic effect.

Besides the antibacterial activity, the antiviral property of rALF*Pm3* against the most severe pathogen in shrimp, the white spot syndrome virus (WSSV), was recently elucidated. WSSV propagation was significantly reduced after rALF*Pm3* was added together with WSSV into the crayfish hematopoietic cell cultures. The rALF*Pm3* also exhibited WSSV neutralization activity resulted in inhibition of WSSV replication in *P. monodon* (Tharntada *et al.,* 2009). In addition, the rALF*Pm3* was shown to be significantly active against non-shrimp pathogenic DNA viruses: herpes simplex virus types 1 (HSV-1) and human adenovirus respiratory strain (AdV-5), in mammalian cell lines (Carriel-Gomes *et al.,* 2007).

CONCLUSIONS

From the current information, it has been found that diverse classes of AMPs are produced in *P. monodon*. The target specificity and strength of antimicrobial activity are diverged among the AMPs. Variable in the expression pattern in response to infection has been observed among AMPs indicating perhaps the different mechanisms of antimicrobial action. These AMPs play crucial roles in shrimp innate immunity enabling the shrimp to survive in nature that normally contains the varieties of pathogenic microorganisms.

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Applications of shrimp immune DNA microarray in aquaculture

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ABSTRACT

Infectious disease constitutes a major obstacle to the sustainability of shrimp aquaculture worldwide and a significant threat to natural populations of shrimp and other crustaceans. The study of the shrimp immune system, including the response to viral infection, has been hampered by a relative lack of molecular genetic information and of tools suitable for high throughput assessment of gene expression. The aim of this paper is to provide insights into the transcriptomic profile on two culture shrimps *Penaeus monodon* and *P. vannamei* responses to immune challenge. In this report, the generation of a tiger shrimp immune DNA microarray encompassing 30 immune-related genes and a cDNA microarray encompassing 6000 putative unigenes expressed in gills, circulating haemocytes, and hepatopancreas of *P. vannamei* is described. Penaeid shrimp immune microarray was applied in screening high disease-resistant broodstocks of tiger shrimp. The result indicated that higher mRNA level *hemocyanin* was found in disease-resistant shrimp broodstocks. The second application of shrimp immune DNA microarray was in nutrigenomic study. The result revealed that the expression of several immune genes (c*rustin*, *lysozyme, Mo-penaeidin*, *transglutaminase* and *Kazal-type proteinase inhibitor*) were increased significantly after injection of microbial immune elicitors (such as lipopolysaccharide, β-1-3 glucan and peptidoglycan) or other immune stimulants (such as polyI:C, Chinese medicine herbs). Shrimp immune DNA microarray analysis may provide useful clues to investigate the intracellular regulation and cross talk between many innate immune genes. Furthermore, shrimp immune DNA microarray might also provide a useful tool to identify disease resistant gene markers for marker-assisted selection program and to explore the Chinese herbs as immune stimulants for paneid shrimps.

Key words: cDNA microarray, innate immunity, penaeid shrimps, immunostimulants

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INTRODUCTION

The shrimp aquaculture with its great potential to generate high export earnings has developed rapidly since 1970s as an industrial activity, but its development was regularly and seriously affected by the outbreak of viral and bacterial diseases during the last decade. From 1993 to 2000, white spot syndrome virus (WSSV), the most devastating viral pathogen of penaeid shrimp (*Penaeus* spp.), caused severe losses to the shrimp farming industry from Asia to America. Therefore, preventing and controlling the spread of disease has become a priority to the shrimp industry.

Host defense in invertebrates including shrimp is believed to rest entirely on innate, non-adaptive immunity that consists of cellular and humoral reactions. Cellular reactions involve phagocytosis, nodule formation and encapsulation, while humoral reactions involve the prophenoloxidase-activating cascade and immune-related proteins such as lysozymes, lectins, and antimicrobial peptides. Our knowledge about the innate immunity of shrimp comes mostly from the studies on crustaceans against bacteria and fungi. Two antivirus protein-pmAVP and hemocyanin from penaeid shrimp have been identified recently, however, the profile of immuno-related factors whereby shrimp defends against virus infection still remains unclear.

cDNA microarrays have emerged as a versatile technology that can be applied with relative ease to studies of differential gene expression in a wide variety of organisms. In present study we try to use of a cDNA microarray to study immunity against viral infection in host following various treatments, it can provide the information of host pathogen interactions, it also can determine which genes are expressed, and in what relative amounts during the infection process.

Nutrigenomics is a science of how the genes interact with nutrients. It is the study of how DNA and the genetic code affect the need for certain nutrients and help maintain optimal health throughout the life. Expression profiling using microarrays offers a powerful tool to gain a comprehensive view of biological systems by measuring the expression of thousands of genes simultaneously. The main advantage of such an approach is the ability to retain the power of microarray technology for monitoring a set of genes selected for their involvement in a specific pathway.

In this study, a cDNA microarray was used to identify genes upregulated in virus-resistant shrimp and to identify their expression profile. Some of these genes that might be involved in defense against virus were identified in the hemolymph of penaeid shrimp. The development of immunostimulants (as green feed additives) to reduce disease susceptibility, a traditional Chinese herb medicine complex formula was used to identify genes expression profile and up- and down-regulated immune-related genes.

MATERIALS AND METHODS

Preparation of WSSV-resistant shrimp

Forty shrimp, *Penaeus monodon* (Crustacea, Decapoda) (about 20–25 g), were obtained from a shrimp farm in PinTon county, Taiwan, and cultured in our laboratory in 500 l tanks (at 25 °C) filled with air-pumped circulating sea water. In order to obtain virus-resistant shrimp, WSSV challenge test was performed. A WSSV inoculum was prepared with virus-infected shrimp tail muscle that tested positive by PCR. Frozen infected tissue was homogenized in 1X PBS (1:5, wt/vol) and centrifuged at 5000 rpm for 10 min. The supernatant was filtered through a 0.45-μm pore-size filter and used for injecting the animals. The surviving shrimps were injected with100 μl of WSSV inoculums between the second and third tail tarsal plates on the lateral side of the shrimp with a 1-ml sterile syringe. Shrimps were challenged with WSSV three times for the period of one month. The 2–3 survivals were collected as WSSV-resistant shrimp. The unchallenged shrimps were used as control (virus-sensitive) shrimp.

Pacific white shrimp (*P. vannamei*) weighing 15-20 g were obtained from a local shrimp farm in I-Lan county, Taiwan. The shrimps were divided into each group of 30 animals. Each was assigned randomly to the three dietary treatments. Each shrimp was housed individually in an 18 l glass tank containing 12 l water. Constant aeration was provided in all tanks during the experiment. Temperature of culture water was approximately 25° C. The shrimps were fed the test diets twice daily throughout the 3 day feeding trial. Six shrimps from each treatment were sampled at 0 h (after feeding), 12 h, 24 h and 72 h (3 days) after the feeding trial began. The moulting stage of each sampled shrimp was determined. Only those in the intermolt or early premolt stages were evaluated in the study.

Chinese herbs compound feeds preparation

Two kinds of Chinese herbs compound (the principal constituents were: *Astragalus membranaceus* Bge, *Lonicera japonica* Thunb, *Isatis indigotica* Fort and *Glycyrrhiza glabra* L.) were added as a solution into normal feeds; for each 1 kg feed 150 ml Chinese herbs solution were added.

Construction of shrimp immune-related DNA microarrays

A *P. monodon* shrimp cDNA microarray that contained 30 unique EST clones was constructed from from cDNA library of *P. monodon* prepared from haemocytes (Table 1). Each EST was spotted in quarters.

The *P. vannamei* oligonucleotide microarray of 5885 elements was constructed. The genes spotted onto the microarray from the haemocytes cDNA library, including the accession number of ESTs, have been submitted to the NCBI GEO. The clones of the unique genes from ESTs were selected based on bioinformatics analysis of the haemocytes ESTs. The probes were design and spotted by Agilent Technologies. Each EST and gene were spotted in duplicate.

Source of samples for microarray hybridization

Two groups of samples were set for hybridization, each containing experimental shrimps and control, to prepare the haemocytes for challenge, Haemolymph was collected from the shrimp by inserting a 26-gauge needle into the ventral sinus cavity and withdrawn into a syringe rinsed by precooled anticoagulant solution (0.1 M sodium citrate, 0.25 M sucrose, 0.01 M Trise HCl, pH 7.6). The diluted haemolymph was centrifuged at 3 000 rpm and 4° C for 15 min to separate the haemocytes from the plasma. The resulting haemocytes pellet was washed with the anticoagulant solution and suspended in Trizol (Invitrogen, Carlsbad, CA) for total RNA isolation.

For *P. monodon* experiment groups to be injected with lipopolysaccharide, peptidoglycan and β-1,3-glucan, each shrimp was injected with 1μg immunostimulant per 1 g og shrimp weight. Haemolymph was collected after injection 48 h for microarray analysis. For *P. vannamei* experimental group to be with injected Poly I:C, each shrimp was injected with 1μg immunostimulant per 1 g of shrimp weight. Haemolymph was collected after injection 0 h, 12 h, 24 h, 48 h and 72 h for semiquantitative RT-PCR. The feeding experiments have two groups for Chinese herbs compound. Each 1 kg feed was added with 150 ml Chinese herbs solution. The shrimp were fed the test diets twice daily throughout the 3 day feeding trials. Uneaten food and excreta were removed each morning before feeding, and the haemolymph was collected after feeding 0 h, 12 h, 24 h, 48 h and 72 h for microarray and semiquantitative RT-PCR.

Microarray analysis

The cDNA transcribed from 20 μg of total RNA from 10 individual *P. monodon* was labeled with Cy3 or Cy5 using a SuperScript™ Plus Indirect cDNA Labeling System (Invitrogen). The arrays were hybridized with the labeled cDNA sample pairs (one with Cy3 and the other with Cy5) overnight at 42° C. After hybridization, the arrays were washed at 30° C in 5X SSC/0.1% (w/v) SDS for 10 min, then twice in 0.5X SSC for 2 min each, and finally briefly in 0.5X SSC/0.01% (v/v) Tween 20. The arrays were scanned using a GenePix 4000B, and the raw images were analyzed with GeneSpring Analysis Platform. After normalization, the values of spot replicates were averaged. A gene was considered differentially expressed by each experiment if its expression ratio increased or decreased by 2-fold or more compared to the control samples.

Semi-quantitative RT-PCR

Total RNA was extracted from a pool of hemocyte pellets using TRI reagent (Invitrogen). The first-strand cDNA was prepared from total RNA with primer. Briefly, the total RNA (5 μg) was mixed with 0.5 μg of oligo dT. RNase-free water was used to make a final volume of 10 μL. The mixture was heated at 65° C for 2 min and then cooled immediately on ice. Thereafter, the volume was adjusted to 20 μL using 10 reaction buffer containing 3 mM $MgCl_2$, 0.5 mM dNTPs, 3 U MMLV reverse transcriptase (EPICENTRE Biotechnologies, USA) and RNase-free water. The first-strand cDNA was then used as the template for PCR amplification with the appropriate primers designed by the Primer Premier 5 of immune-related genes. Semi-quantitative RT-PCR analysis was performed on total RNA isolated

from control and infected hemocyte at various time-points. RT-PCR of elongation factor-1 was performed and used for normalization of total RNA variation. RNA expression levels were quantitatively analyzed using Kodak Electrophoreisis Documentation and Analysis System 290 (Kodak EDAS 290).

Data analysis

A multiple comparison (Tukey) test was conducted to compare the significant difference for each gene expression among different time courses using a SAS computer software (SAS Institute Inc., Cary, NC, USA). A significant level of $p = 0.05$ was chosen.

RESULTS

In order to find the immune-relevant factors responsible for the virus resistance in the WSSV-resistant shrimp, a cDNA microarray method was employed to identify differentially expressed genes and their expression profile in the hemolymph of the virus-resistant penaeid shrimp. Twenty five genes were identified from more than 8000 clones, of which are related to innate immunity in penaeid shrimp.

Application of penaeid shrimp immune cDNA microarray in screening high disease-resistant broodstocks of tiger shrimp

Hemocyanin is the most abundant gene in our cDNA microarray analysis except penaeidin, crustin, lectin, ferritin, oxygenase and chitinase of the virus-resistant black tiger shrimp; all showed up-regulation in expression compared with those of normal. The result indicated that higher mRNA level *hemocyanin* and *5HT1-receptor* were found in disease-resistant shrimp broodstocks (Fig.1).

Shrimps followed by feeding of WSSV-containing tissue at 10 % biomass daily for 3 consecutive days or injected intramuscularly with 100 µl WSSV inoculums (10^{-6} dilution) resulted in a cumulative >80% death of the tiger shrimps at 4 days post infection (Fig. 2a, 2b). Control shrimp injected with dsRNA (shrimp hemocyanin siRNA) showed higher mortality from the control at 2 days following both viral infection methods. On the other hand, WSSV-resistant shrimp fed with 3% complex herbs formula showed increase in survival rate from the control start from 4 days post infection. No shrimp death was observed through the whole experimental period, indicating that shrimp hemocyanin siRNA and complex Chinese herbs formula did not give any cytotoxicity.

Application of shrimp immune cDNA microarray in nutrigenomic study

After the treatment with Chinese herbs complex formula, the hemolymph were collected to isolate total RNA and analyzed using the immune-related cDNA microarray which contained approximately 6 000 shrimp genes. Chinese herbs changed the expression of the genes related to immune response, cell growth and cell proliferation, signal transduction in *P. vannamei*. In this study, up-regulation of innate immunity genes was the most remarkable and it was suggested the mediation of *crustin*, *lysozyme*, *Mo-penaeidin*, *transglutaminase*

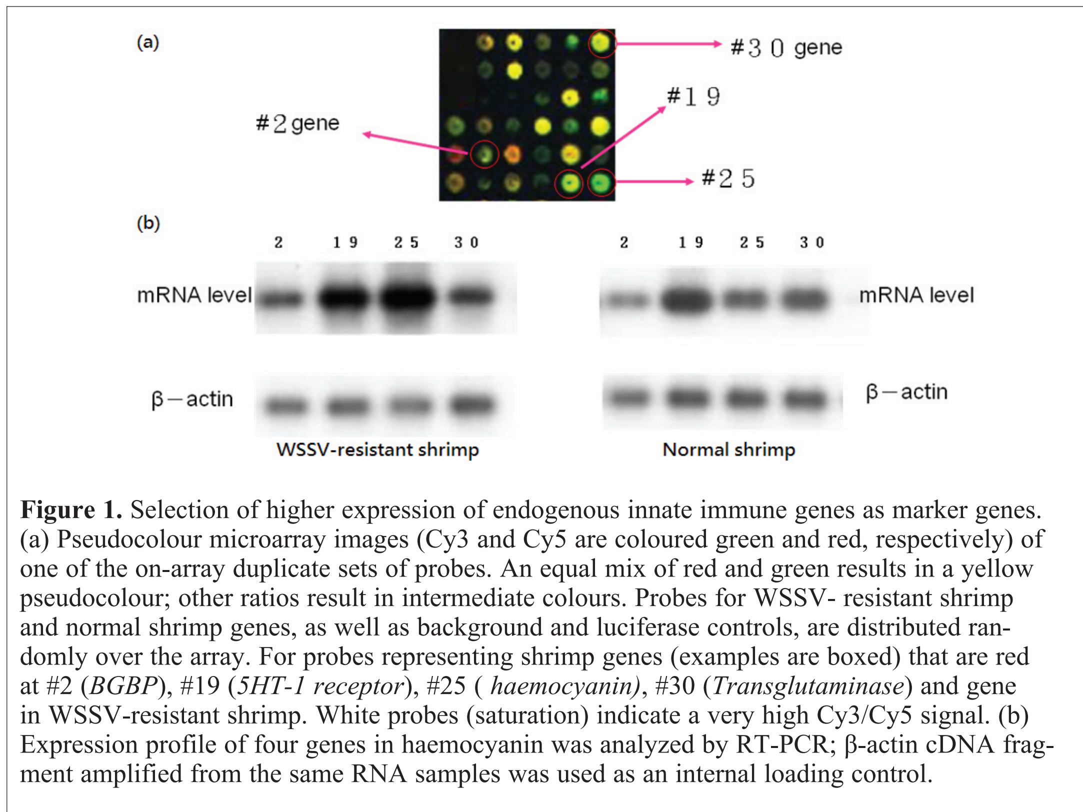

Figure 1. Selection of higher expression of endogenous innate immune genes as marker genes. (a) Pseudocolour microarray images (Cy3 and Cy5 are coloured green and red, respectively) of one of the on-array duplicate sets of probes. An equal mix of red and green results in a yellow pseudocolour; other ratios result in intermediate colours. Probes for WSSV- resistant shrimp and normal shrimp genes, as well as background and luciferase controls, are distributed randomly over the array. For probes representing shrimp genes (examples are boxed) that are red at #2 (*BGBP*), #19 (*5HT-1 receptor*), #25 (*haemocyanin)*, #30 (*Transglutaminase*) and gene in WSSV-resistant shrimp. White probes (saturation) indicate a very high Cy3/Cy5 signal. (b) Expression profile of four genes in haemocyanin was analyzed by RT-PCR; β-actin cDNA fragment amplified from the same RNA samples was used as an internal loading control.

Table 1. Innate-immune related EST tags of tiger shrimp

Gene Name	Gene Name
1: Anti-lipopolysaccharide factor (ALF)	16: Intracellular fatty acid binding protein
2: Beta-1,3-glucan binding protein (BGBP)	17: Kazal-type proteinase inhibitor
3: 11.5 kDa antibacterial peptide	18: Glutathione peroxidase
4: Prophenoloxidase	19: 5-HT1 receptor
5: Prophenoloxidase activating factor	20: Cytochrome b
6: Peroxinectin	21: Innexin 1
7: Serine proteinase	22: α- tubulin
8: α-2-macroglobulin	23: Ribosomal protein L27a
9: Cytosolic manganese superoxide dismutase	24: RNA virus putative RNA-dependent RNA polymerase
10: Lysozyme	25: Hemocyanin
11: Chelonianin	26: Syntenin
12: PMAV (virus resistance)	27: Mo-penaeidin
13: Astakine	28: Clottable protein
14: Heat shock protein 90	29: Heat shock protein 70
15: Heat shock protein 10	30: Transglutaminase

Table 2. 2x expression up and down from β-glucan in *Penaeus monodon*

Gene Name	Normalized Data	Gene Name	Normalized Data
11.5 kDa antibacterial peptide (Crustin)	37.61	Cytosolic manganese superoxide dismutase	0.399
Kazal-type proteinase inhibitor	10.27	Astakine	0.0594
Prophenoloxidase activating factor	9.111	RNA virus putative RNA-dependent RNA polymerase	0.0399
ribosomal protein L27a	7.016	Serine proteinase	0.0355
Mo-penaeidin	6.851	Heat shock protein 10	0.0304
beta-actin	5.714	syntenin	0.0278
Lysozyme	5.163	Peroxinectin	0.0277
alpha-2-macroglobulin	4.982	Beta-1,3-glucan binding protein (BGBP)	0.0276
cytochrome b	3.9	Intracellular fatty acid binding protein	0.01
Anti-lipopolysaccharide factor (ALF)	3.735	Clottable protein	0.01
Transglutaminase	3.663	PMAV (virus resistance)	0.01
beta-actin	3.237	hemocyanin	0.01
Chelonianin	2.629		
5-HT1 receptor	2.034		

Table 3. 2x expression up and down from peptidoglycan in *P. monodon*

Gene Name	Normalized Data	Gene Name	Normalized Data
11.5 kDa antibacterial peptide (Crustin)	2.733	5-HT1 receptor	0.151
Heat shock protein 90	0.363	Peroxinectin	0.148
alpha-tubulin	0.36	Astakine	0.139
Prophenoloxidase activating factor	0.356	syntenin	0.125
Glutathione peroxidase	0.303	Clottable protein	0.105
Prophenoloxidase	0.268	hemocyanin	0.102
Heat shock protein 10	0.229	RNA virus putative RNA-dependent RNA polymerase	0.0982
Serine proteinase	0.201	Beta-1,3-glucan binding protein (BGBP)	0.0685
PMAV (virus resistance)	0.178	5-HT1 receptor	0.151
innexin 1	0.175	Peroxinectin	0.148
Intracellular fatty acid binding protein	0.167		

Table 4. 2x expression up and down from lipopolysaccharide in *P. monodon*

Gene Name	Normalized Data	Gene Name	Normalized Data
11.5 kDa antibacterial peptide (Crustin)	5.314	Cytosolic manganese superoxide dismutase	0.255
Lysozyme	4.326	Astakine	0.219
cytochrome b	3.55	Intracellular fatty acid binding protein	0.189
Peroxinectin	0.406	Heat shock protein 10	0.147
innexin 1	0.357	Beta-1,3-glucan binding protein (BGBP)	0.145
Glutathione peroxidase	0.347	syntenin	0.108
Prophenoloxidase	0.326	5-HT1 receptor	0.0995
alpha-tubulin	0.296	PMAV (virus resistance)	0.0772
Serine proteinase	0.293	RNA virus putative RNA-dependent RNA polymerase	0.0757
Heat shock protein 90	0.268	Clottable protein	0.0692
hemocyanin	0.0427		

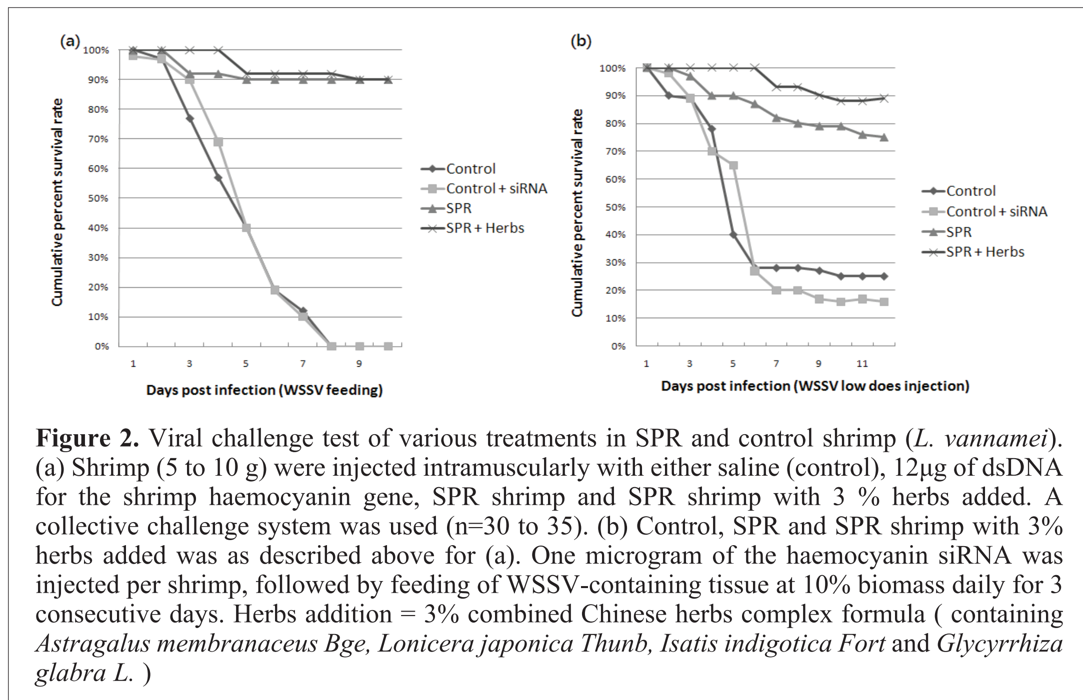

Figure 2. Viral challenge test of various treatments in SPR and control shrimp (*L. vannamei*). (a) Shrimp (5 to 10 g) were injected intramuscularly with either saline (control), 12μg of dsDNA for the shrimp haemocyanin gene, SPR shrimp and SPR shrimp with 3 % herbs added. A collective challenge system was used (n=30 to 35). (b) Control, SPR and SPR shrimp with 3% herbs added was as described above for (a). One microgram of the haemocyanin siRNA was injected per shrimp, followed by feeding of WSSV-containing tissue at 10% biomass daily for 3 consecutive days. Herbs addition = 3% combined Chinese herbs complex formula (containing *Astragalus membranaceus Bge, Lonicera japonica Thunb, Isatis indigotica Fort* and *Glycyrrhiza glabra L.*)

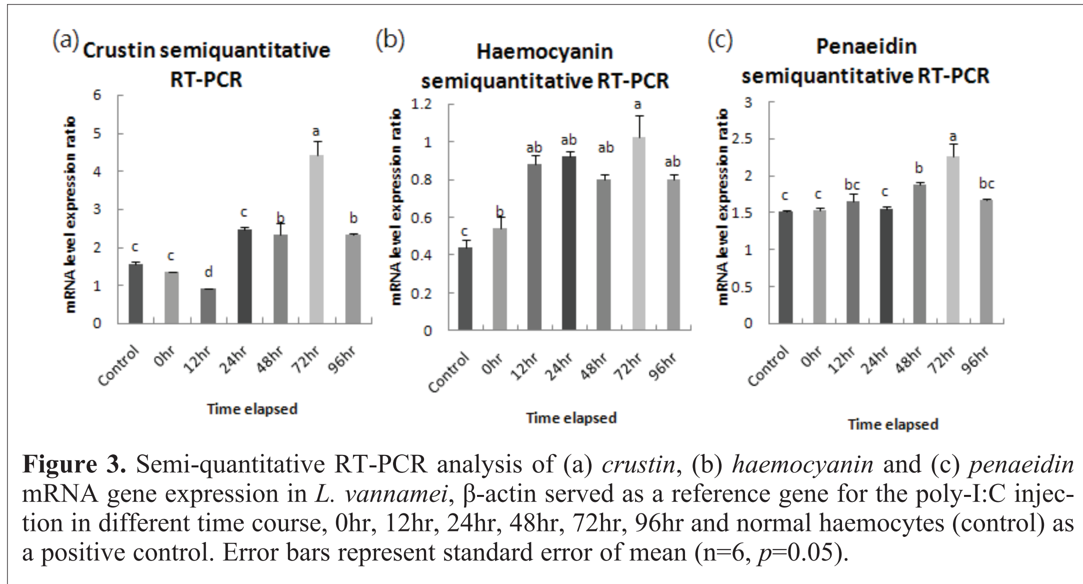

Figure 3. Semi-quantitative RT-PCR analysis of (a) *crustin*, (b) *haemocyanin* and (c) *penaeidin* mRNA gene expression in *L. vannamei*, β-actin served as a reference gene for the poly-I:C injection in different time course, 0hr, 12hr, 24hr, 48hr, 72hr, 96hr and normal haemocytes (control) as a positive control. Error bars represent standard error of mean (n=6, p=0.05).

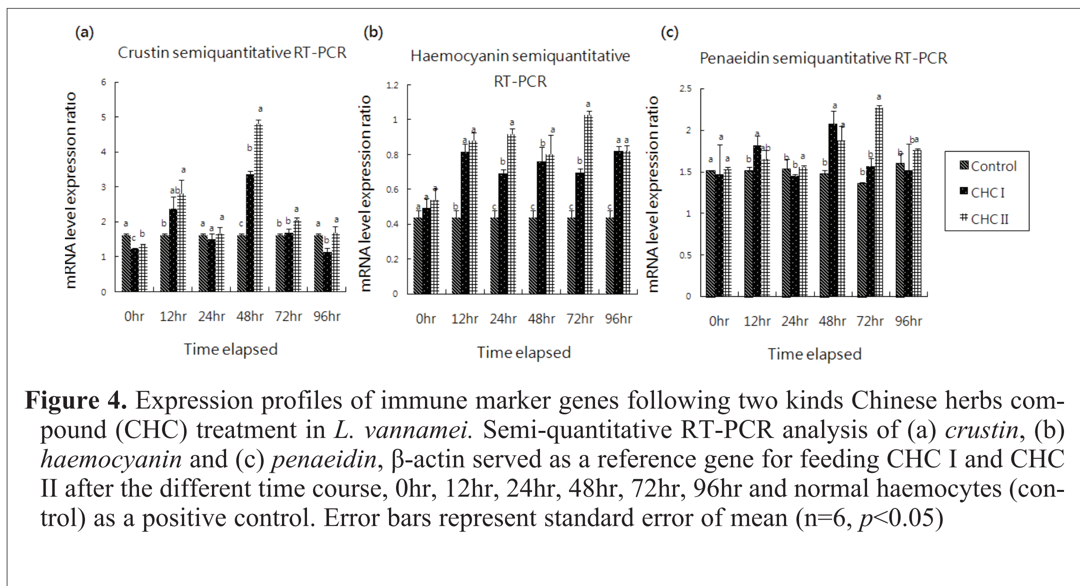

Figure 4. Expression profiles of immune marker genes following two kinds Chinese herbs compound (CHC) treatment in *L. vannamei.* Semi-quantitative RT-PCR analysis of (a) *crustin*, (b) *haemocyanin* and (c) *penaeidin*, β-actin served as a reference gene for feeding CHC I and CHC II after the different time course, 0hr, 12hr, 24hr, 48hr, 72hr, 96hr and normal haemocytes (control) as a positive control. Error bars represent standard error of mean (n=6, $p<0.05$)

and *Kazal-type proteinase inhibitor* responded significantly by microbial immune elicitors (such as lipopolysaccharide, β-1-3 glucan and peptidoglycan) (Tables 2, 3, 4) and other immune challenges (such as polyI:C , Chinese medicine herbs) treatment in black tiger shrimp and Pacific white shrimp (Fig. 3 and Fig. 4). On the other hands, Chinese herbs also up- and down-regulated the mRNA expression of some immune-related genes (Fig. 5). The data also gave new information about the regulation by Chinese herbs in penaeid shrimp.

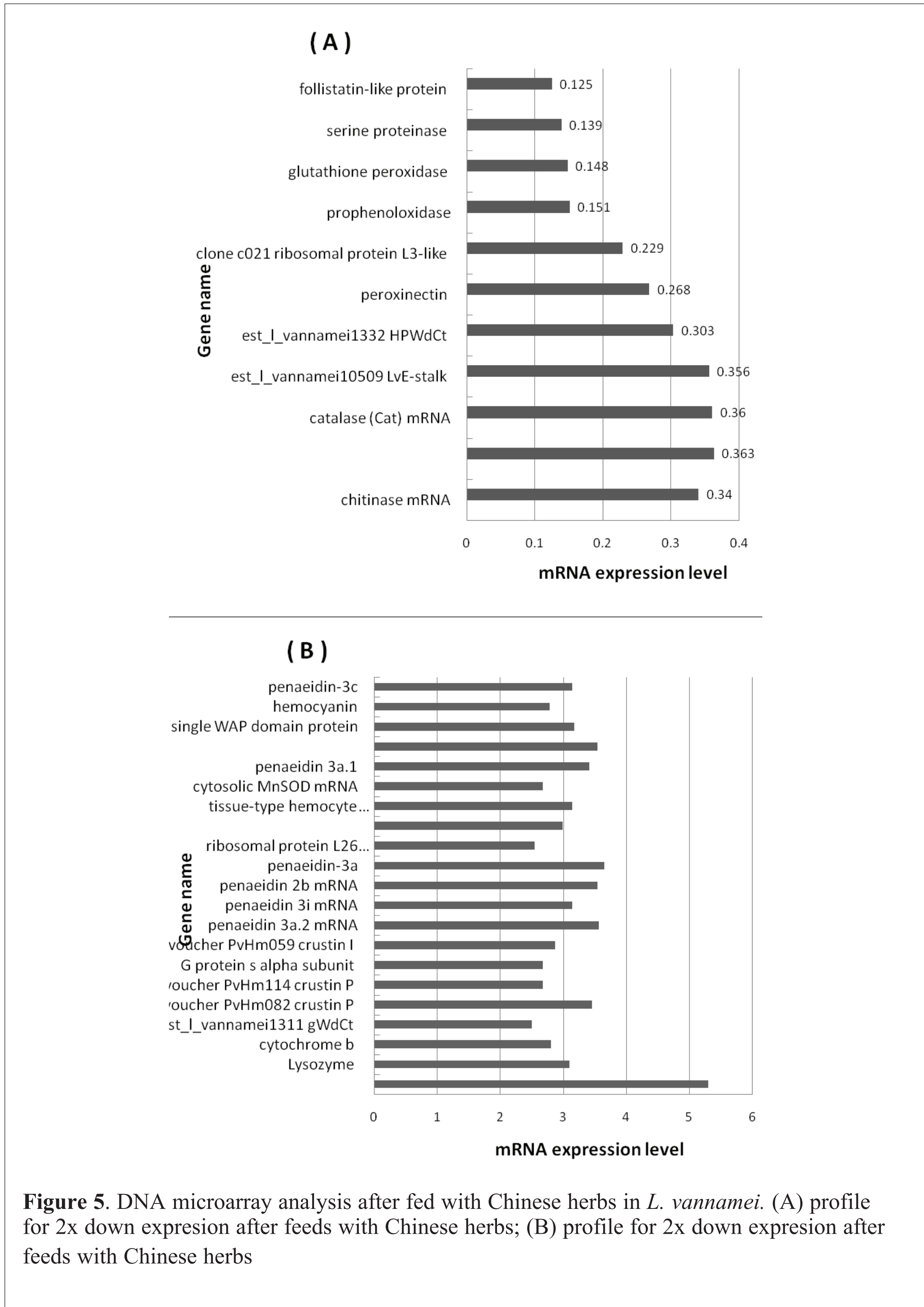

Figure 5. DNA microarray analysis after fed with Chinese herbs in *L. vannamei.* (A) profile for 2x down expresion after feeds with Chinese herbs; (B) profile for 2x down expresion after feeds with Chinese herbs

DISCUSSIONS

DNA microarray analysis could provide useful clues to investigate the intracellular regulation and cross talk between many innate immune genes and regulators, and also to explore the novel function of Chinese herbs.

Control shrimp injected with dsRNA (shrimp hemocyanin siRNA) showed higher mortality from the control at 2 days following both viral infection methods demonstrating that the hemocyanin might serve a role in protection against WSSV (Fig. 2a). The WSSV-resistant shrimp fed with 3% complex herbs formula showed increase in survival rate from the control start from 4 days post infection demonstrating that WSSV-resistant shrimp and herb addition gave a better protection against WSSV infection (Fig. 2b).

The results of present study indicated that the hemolymph is a crucial system in the immune system of penaeid shrimp and cDNA microarray is an effective approach for discovering immune relevant genes. Moreover, several immune related genes (such as c*rustin*, *lysozyme, Mo-penaeidin*, *transglutaminase* and *Kazal-type proteinase inhibitor*) showed significant up-regulation in the virus-resistant shrimp suggesting that these genes may play an important role in the virus defense response of penaeid shrimp. Transcription-based data mining of genes in QTL-limited intervals followed by efficient quantitative qRT-PCR or semi-quantitative RT-PCR methods is an effective strategy for identifying genes that may contribute to complex shrimp defense processes. After viral infection, a number of genes whose expression is altered, and thus are candidates for disease-resistant QTL and/or pathways associated with shrimp defense system. The results also confirmed that the data from cDNA microarray analysis could be correlated with the *in vivo* effect of the immune-enhancing compound (Rattanachai *et al.,* 2005; Wang, 2008).

In the present study, we have demonstrated that *hemocyanin, crustin*, *lysozyme* and *Mo-penaeidin* can serve as marker genes for screening disease-resistant shrimp broodstocks and to exploit novel immunostimulants as aquafeed additives. Here, we describe how nutrigenomics could provide new insights into animal nutrition research and innovative developments through neutraceutical products in penaeid shrimps.

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Molecular immunity in the interaction between fish and pathogen for DNA vaccine

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ABSTRACT

Cultured fish are threatened by many pathogens, especially viruses and bacteria, often with serious consequences. Vaccination is one of the most effective tools for enhancing host defense and protecting fish from pathogens. DNA vaccines are a third generation vaccine based on the administration of the gene encoding a vaccine antigen rather than the antigen itself. To date, several effective DNA vaccines that encode viral glycoproteins or other antigenic proteins have already been shown to be effective for cultured fish. This review summarizes current knowledge on fish viral pathogens and DNA vaccines against fish viral diseases, especially against hirame rhabdovirus (HIRRV), viral hemorrhagic septicemia virus (VHSV) and red seabream iridovirus (RSIV) from previous studies. Furthermore, the mechanism of interaction between the DNA vaccines and host immunity is described using mammalian evidence and data gained from using our Japanese flounder microarray chip. The efficacy of two DNA vaccines derived from pathogenic viruses such as HRV and VHSV have been evaluated through gene expression profiles. A comparison of gene expression profiles of vaccinated and unvaccinated fish suggests important evidences that DNA vaccines have a role in host immunity such as induction of MHC class I gene expression and T-cell stimulation.

Keywords: DNA vaccine, Japanese flounder (*Paralichthys olivaceus*), microarray, VHSV, HIRRV

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INTRODUCTION

Fish supplied 110 million tons of food in 2006, of which 47% (51.7 million tons) was supplied by aquaculture (FAO, 2009). The aquaculture industry grew at an amazing rate of 7.6% from 2000-2006 with an extreme growth rate of almost 40% for Vietnam (Brugère and Ridler, 2004). The industry is expected to grow more, to meet the demand of a growing global population. Japanese flounder (*Paralichthys olivaceus*, also known as olive flounder or Bastard halibut) is a major aquaculture product in Japan, Korea, and China. However, recently, the spread of an increasing array of new diseases cause epizootics that result in substantial socio-economic and environmental losses (Walker and Winton, 2010).

Fish pathogenic viruses

One of the biggest problems in aquaculture is disease caused by viruses. The OIE (World Organisation for Animal Health, originally called the Office International des Epizooties, thus its acronym) is the intergovernmental organization responsible for improving animal health worldwide (http://www.oie.int). It keeps a list of 'Fish diseases notifiable to the OIE' which means these diseases are considered to be of socio-economic and/or public health importance within countries, and significant to the international trade in aquatic animals and aquatic animal products. Of the nine fish diseases listed in 2010 by the OIE as notifiable, seven are viral. These are mostly members of the Rhabdoviridae and Iridoviridae family and they have caused tremendous socio-economic losses worldwide.

Epizootic haematopoietic necrosis (EHNV: Unassigned, *Iridoviridae, Ranavirus*) causes severe necrosis of the haematopoietic tissue of perch and rainbow trout (Whittington *et. al*., 1996; Reddacliff and Whittington, 1996).

Infectious haematopoietic necrosis (IHNV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) causes the hemorrhage of haematopoietic tissue and nephron cells leading to death by edema. It has caused mass mortalities of salmon and trout and occurs at low water temperatures (8-15°C) (McAllister *et al*., 1974; Holway and Smith, 1973).

Spring viraemia of carp (SVCV: *Mononegavirales, Rhabdoviridae, Vesiculovirus*) is characterized by external and internal hemorrhages, peritonitis and ascites. It predominantly affects common carp (*Cyprinus carpio*) (Essbauer and Ahne, 2001).

Viral haemorrhagic septicaemia (VHSV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) is known as one of the most serious viral pathogens of salmonids and marine fishes in the world (Mortensen *et al*., 1999). It has been known to infect eels, herring, carp, cod, perch, flatfish, salmon, and flounder (Isshik *et al.,* 2001; Dopaz *et al.,* 2002). It also occurred in wild Japanese flounders during a survey on the distribution of fish viruses in wild marine fishes in Japan (Takano *et al*., 2000). The virus causes hemorrhagic lesions in internal organs and muscular tissue. The virus multiplies in endothelial cells, leucocytes, hematopoietic tissues and nephron cells.

Infectious salmon anaemia (ISAV: Unassigned, *Isavirus*) is often associated with an haemorrhagic kidney disease (Lovely *et al*., 1999). ISAV has caused severe economic losses to Atlantic salmon cultured in Europe, North America, and Chile (Rodger and Richards, 1998; Falk *et al*., 1997; Mjaaland *et al*., 1997).

Iridoviruses, including Red seabream iridoviral disease (RSIV: *Iridoviridae*) are large icosahedral cytoplasmic DNA viruses that have been isolated from a diverse number of invertebrate and vertebrate hosts (Williams *et al*., 2000). RSIV has caused significant economic losses in aquaculture. This disease was first observed in red seabream (*Pagrus major*) cultured in Shikoku Island, Japan in 1990 (Matsuoka et. al., 1996). A survey on the extent of RSIV infection showed that this virus infects 31 marine fish species including 28 Perciformes, two Pleuronectiformes and one Tetraodontiformes (Kawakami and Nakajima, 2002). Gross pathologies can include severe anemia, petechia of the gills and enlargement of the spleen.

Koi herpesvirus disease (KHV: *Herpesvirales, Alloherpesviridae, Cyprinivirus*) is characterized by papillomas on the caudal fin region. KHV has caused enormous losses around the world (Bretzinger *et al.*, 1999; Ariav *et al*., 1999).

Aside from these major viral diseases, several other viruses have caused much damage to fish aquaculture species around the world (Table 1). Some of the most notable are:

Viral nervous necrosis virus (VNNV: Unassigned, *Nodaviridae, Betanodavirus*) can infect several fish species (Tanaka *et al.,* 2003). It induces abnormal swimming behavior, encephalopathy and retinopathy. Since it was first discovered, several similar cases have been reported around the world. One of the most prominent species is the striped jack viral nervous necrosis virus (SJNNV).

Hirame rhabdovirus (HIRRV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) is mostly associated with epizootics and heavy losses in aquaculture (Kimura *et al*., 1986) and is primarily a pathogen of Japanese flounder. It is composed of five structural proteins with similarity to those of IHNV and VHSV (Nishizawa *et al*., 1991). Transmission occurs mainly by shedding from infected fish, and the viruses are spread by waterborne contact (Wolf, 1988). The early targets for the viruses are the gills, the esophagus, cardiac stomach region, and mucus-secreting glands.

Lymphocystis disease virus (LCDV: Unassigned, *Iridoviridae, Lymphocystivirus*) causes transformation and enlargement of cells of the skin and in the connective tissue of internal organs. Infected cells undergo massive hypertrophy and encapsidation by an extracellular hyaline matrix. LCD is characterized as a chronic benign disease with rare mortality. Infection rates are increased by stress factors (Wolf, 1988).

Marine birnavirus (MABV: Unassigned, *Birnaviridae, Aquabirnavirus*) has been isolated in flounders (Jung *et al*., 2008). It causes accumulation of ascitic fluid in the abdominal

Table 1.
Viral Families causing major diseases in fish

Group[a]	Order[b]	Family	Representative Species
A. DNA Viruses			
I: dsDNA	Caudovirales/	Iridoviridae	Epizootic haematopoietic necrosis virus (EHNV)*
	Herpesvirales		Red seabream iridovirus (RSIV)*
			Lymphocystis disease viruses (LCDV)†
		Herpesviridae	Koi herpesvirus (KHV, or CyHV-3)*[c]
			Channel catfish herpesvirus (CCHV)
B. RNA Viruses			
III: dsRNA		Reoviridae	Golden shiner reovirus (GSRV)
			Grass carp reovirus (GCRV)
		Birnaviridae	Infectious pancreatic necrosis virus (IPNV)
			Marine fish birnaviruses (MABV)†
IV: ss(+)RNA	Nidovirales /	Caliciviridae	San Miguel sea lion virus (SMSV)
	Picornavirales	Togaviridae	Salmon pancreatic disease virus (SPDV)
			Sleeping disease virus of rainbow trout (SDV)
		Nodaviridae	Viral nervous necrosis virus (VNNV)†
V: ss(-)RNA	Mononegavirales	Orthomyxoviridae	Eel viruses (A1B, EV1 and EV2)
			Infectious salmon anaemia virus (ISAV)*
		Rhabdoviridae	Infectious haematopoietic necrosis virus (IHNV)*
			Viral haemorrhagic septicaemia virus (VHSV)*†
			Spring viraemia of carp virus (SVCV)*
			Hirame rhabdovirus (HIRRV)†
VI: RT-Transcribing		Retroviridae	Walleye dermal sarcoma virus (WDSV)

*OIE listed disease

†Infectious to *P. olivaceus*

[a] Viral Groups are arranged according to Baltimore classification (I, III-VI)

[b] Viruses are classified using ICTV nomenclature

[c] KHV is classified as belonging to the Alloherpesviridae family using ICTV classification

cavity, congestion in the liver and absence of food in the intestine with hemorrhages. External signs can be hemorrhages on the body surface and white nodules in the kidney and spleen.

Advantages of vaccine use

Several strategies have been implemented to alleviate the damage caused by these diseases. One of the most commonly used strategy against disease, in general, is chemotherapy by using antibiotics. However, there is cause for serious concern regarding the overutilization of antibiotics, which can lead to serious environmental damage by upsetting the natural microbial population and can hasten the emergence of antibiotic resistant pathogens (Huovinen, 1999a; Huovinen, 1999b; Park *et al*., 2009). Moreover, antibiotics are not effective against viruses. Therefore, an effective strategy against viral pathogens must be considered. One of the most promising techniques is vaccination (Gudding *et al*., 1999; Hastein *et al*., 2005).

The principal difference between antibiotics and vaccines is that antibiotics are a therapeutic strategy, to be administered upon the onset of a disease. Vaccines, on the other hand, are a preventive strategy, and depend on stimulating the immune system of the target animal. Also, antibiotics are active mostly against bacterial pathogens, and will be effective only if an appropriate concentration is maintained. Vaccines, upon stimulation of the host immune system, need not be maintained, and the duration of protection surpasses that of antibiotics (Grisez and Tan, 2005).

Administration

Vaccines can be delivered orally, by immersion, or by injection. Vaccines to be delivered orally are integrated into the diet (by mixing, top-dressing, or bioencapsulation). Oral administration is the most straightforward and easiest method, but problems with getting the vaccine intact through the digestive system are prevalent. Attempts to improve this method have mostly focused on protecting the vaccine from digestive enzymes (Ellis, 1998). Immersion relies on stimulating the immune response of the skin and gills of the fish to protect it from future infection. It can be done by dipping (higher concentration of the vaccine, shorter exposure time), or bathing (lower concentration, longer exposure) (Nakanishi and Ototake, 1997). Though it is theoretically practical for large amounts of small fish, it can suffer the same problems with oral vaccines. Vaccination by injection is more direct, and the antigenic effect may be stimulated by an adjuvant (Grisez and Tan, 2005). Protection from vaccines delivered by injection can last for 6 months to a year. The vaccine is injected into the muscle (IM) or into the body cavity [intracoelomic (ICe), or intraperitoneal (IP)]. The injection also allows for multiple antigens to be combined into a single vaccine (Evelyn, 2002). However, since all fish must be handled individually, this method requires more time, labor, and skilled personnel.

Vaccine development

Development of vaccines is a time-consuming and laborious process. As with any drug-development process, it initially involves the screening of potential candidates, which will lead to the identification and isolation of the therapeutic agent/vaccine (discovery).

Characterization of the epidemiology and pathogenesis of the etiological agent, in order to identify targets for immune response or key virulence factors, would help in focusing the screening effort, in the enhancement of the potential antigen by genetic engineering, or in the development of DNA constructs for 3rd generation vaccines (DNA vaccines). A challenge model, to facilitate testing, which must show consistently reproducible and significant data, must be developed (Gudding *et al*., 1999). The candidate vaccines must undergo several in vivo and ultimately in vitro trials to test for positive and/or undesirable effects. With the use of genetic engineering techniques, a vaccine candidate can be streamlined to attenuate any undesired traits and/or to increase desired effects. The candidate can then go to 'production process development', wherein the method for large scale production and eventual delivery must be optimized. In all this, potential expenditure for production must be realistically assessed with the potential cost of the final product. This candidate must then undergo stringent tests for quality and safety to pass registration with relevant government agencies. Finally, the product must be marketed. Ideally, the final vaccine should: a) be safe for the fish, the caretaker, and the end-user; b) have a broad and effective protective spectrum; c) provide lasting protection; d) be easy to administer; e) be easy and cost-effective to manufacture; and f) be easily licensed or registered.

Types of vaccines

First generation vaccines utilize the whole pathogen – live, weakened, or killed. These include bacterins, which are composed of killed pathogenic cells that stimulate the humoral (antibody) immune response; and live, attenuated vaccines, which can stimulate both cell-mediated (killer T-cells T_K, helper T-cells, T_H) and antibodies. This is currently the most commonly used type in aquaculture (Hastein *et al*., 2005). The biggest danger is that live pathogens can revert to a dangerous form, while bacterins are ineffective against several pathogens. Second generation vaccines utilizes antigenic subunits of the whole pathogen, such as recombinant protein antigens (toxoids), or other components. These can elicit T_H and antibody responses but not a T_K response. Third generation vaccines or DNA vaccines are directly inoculated DNA constructs that encode a specific antigen under the control of a eukaryotic promoter to stimulate *in vivo* synthesis of immunogenic protein and immune responses. After the vaccinated protein is expressed, it generates an immune response against the DNA-encoded immunogen. This mechanism has been revealed in mammals, when mice were inoculated with plasmids expressing human growth, but developed antibodies instead (Tang *et al*., 1992). The advantage of DNA vaccines is that they have been shown to elicit antibody, T_H, and cytotoxic T lymphocyte (CTL) response. DNA vaccine techniques have been investigated in cultured fish (Lorenzen *et al*., 1998, 1999, 2009; Corbeil *et al*., 1999; 2000) and there is currently tremendous interest in the development of DNA vaccines for fish (Biering *et al*., 2005; Kurath, 2005; Lorenzen and LaPatra, 2005).

DNA vaccines against viral diseases in fish

To date, several DNA vaccines have been examined for use in fish viral diseases (Table 2), especially against viral haemorrhagic septicaemia virus (VHSV), hirame rhabdovirus (HIRRV) and red seabream iridovirus (RSIV).

Table 2.
Summary of DNA vaccines against fish viral diseases

Disease	Antigens	Species	Reference
A. DNA Viruses			
Channel catfish herpesvirus (CCHV)	Several open reading frame (ORF)59, ORF6	Channel catfish	Nusbaum *et al*., 2002
Red seabream iridovirus (RSIV)	Major capsid protein (MCP) and an ORF569	Red seabream	Caipang *et al*., 2006
Lymphocystis disease virus (LCDV)	MCP	Japanese flounder	Tian and Yu, 2010
B. RNA Viruses			
Infectious hematopoietic necrosis virus (IHNV)	Glycoprotein	Rainbow trout,	Anderson *et al*., 1996
		Atlantic salmon	Leong *et al*., 1997
			Winton, 1997
			Corbeil *et al*., 2000
Viral haemorrhagic septicaemia virus (VHSV)	Glycoprotein	Rainbow trout	Boudinot *et al*., 1998
			Heppell *et al*., 1998
			Lorenzen *et al*., 1998
			McLauchlan *et al*., 2003
		Japanese flounder	Byon *et al*., 2005
			Byon *et al*., 2006
Infectious pancreatic necrosis virus (IPNV)	VP2	Brown trout	de las Heras *et al*., 2009
		Rainbow trout	de las Heras *et al*., 2010
Atlantic halibut nodavirus (AHNV)	Coat protein (weakly effective)	Turbot	Sommerset *et al*., 2005
	Glycoprotein of VHSV		Sommerset *et al*., 2003
Hirame rhabdovirus	Glycoprotein	Japanese flounder	Takano *et al*., 2004
Spring viraemia of carp virus (SVCV)	Glycoprotein	Common carp	Kanellos *et al*., 2006
		Koi carp	Emmenegger and Kurath, 2008
Infectious salmon anaemia virus (ISAV)	Hemagglutinin-esterase (HE) (weakly effective)	Atlantic salmon	Mikalsen *et al*., 2005
VHSV and IHNV (Mixed DNA vaccine)	Glycoproteins of VHSV and IHNV	Rainbow trout	Einer-Jensen *et al*., 2009

The glycoprotein (G-protein) gene of VHSV is a highly immunogenic viral protein when used as a DNA vaccine although DNA vaccination with the nucleocapsid (N) protein, phosphoprotein, non-virion, and matrix protein genes of IHNV were shown to be inefficient in rainbow trout (Lorenzen *et al*., 1999; Corbeil *et al*., 1999). Immunization using the VHSV G-protein gene in Japanese flounder, however, showed a high protective efficiency with 93% relative percentage survival (RPS) (Byon *et al*., 2005).

A DNA vaccine encoding HIRRV G-protein gene provided strong protection against HIRRV (Takano *et al*., 2004). Fourteen days post-HIRRV-challenge, the RPSs of fish infected with 1 and 10 μg plasmid DNA vaccine were 70.5 and 90.1%, respectively. However, a DNA vaccine encoding the N-protein gene was inefficient against HIRRV

(Yasuike *et al*., 2010). Interestingly, the gene expression patterns during HIRRV infection between fish vaccinated with the G- and N-protein were substantially different, as shown in the next section.

Vaccination protocols, using formalin-killed virus, have been found to be highly efficient in protecting fish against RSIV (Nakajima *et al*., 1997; Kawakami and Nakajima, 2002). However, the use of whole-killed antigen vaccines has its own limitations, which include poor induction of cell-mediated immunity and poor immunogenicity (Davis and McCluskie, 1999). DNA vaccines encoding the viral major capsid protein (MCP) and an open reading frame (ORF) containing a transmembrane domain have been successfully used against RSIV in red seabream (Caipang et. al., 2006). The RPS of fish treated with the DNA vaccines and their combination ranged from 42.8 to 71.4%. These vaccines significantly induced the expression of MHC class I transcript in the vaccinated fish 15 to 30 days post immunization (Caipang *et al*., 2006).

Immune-related genes in Japanese flounder immunized with DNA vaccines

To understand the immunological response to DNA vaccination, it is necessary for an effective technology to comprehensively analyze the transcripts expressed by the vaccines. Microarrays are specially treated glass slides robotically spotted with thousands of genes (Schena *et al*., 1995). We have previously analyzed the expressions of about 2000 Japanese flounder ESTs with microarray chips (Kurobe *et al*., 2005; Byon *et al*., 2005, 2006; Yasuike *et al*., 2007; Dumrongphol *et al*., 2009). These chips have been used to evaluate the effectiveness of DNA vaccines in Japanese flounder infected with three viral pathogens (such as VHSV and HIRRV).

A microarray analysis of Japanese flounder immunized with DNA vaccine encoding VHSV G-protein was conducted to understand the gene expression patterns of the non-specific and specific immune responses to the vaccination (Byon et. al., 2005, 2006). Non-specific immune response genes such as NK Kupffer cells receptor, MIP1-a and Mx1 protein gene were observed to be up-regulated at 1 and 3 days post-immunization, while the specific immune response genes containing the CD20, CD8 alpha chain, CD40 and B lymphocyte cell adhesion molecule were also up-regulated during that time. These results suggested that the VHSV G-protein gene elicits strong humoral and cellular immune responses, which may play an important role in protecting the fish during viral infections.

Microarray analyses showed differential gene expressions in Japanese flounder in response to DNA vaccination by HIRRV G- and N-proteins (Yasuike *et al*., 2007, 2010). Five genes, including the interferon-stimulated gene 15kDa (ISG15), ISG56, Mx and two unknown genes, were strongly induced after the injection by the HRV G-protein, but not N-protein. The three genes are known as type I interferon (IFN)-inducible genes, which inhibit viral replication or protein synthesis (Caipang *et al*., 2003; Haller and Kochs, 2000; Samuel, 2001; Guo *et al*., 2000), suggesting that stimulation of the type I IFN system protects against HRV infection. Furthermore, a microarray analysis of pHRV-G-vaccinated flounder infected with HIRRV showed up-regulation of several genes within 3 days post-infection

(Yasuike *et al*., 2010). These included genes with homology to mammalian T cell activation-related (such as Cytohesin-1, CXCR3, CARD11/CARMA1, gp96, CaMKII, DAP10, DC-SIGN, PA28α and α2m) and complement system (such as CD59, MASP-2 and complement factor H).

Molecular and cellular interactions between DNA vaccines and host immunity

The schematic pathway of the host immune mechanisms that DNA vaccines effectively activate in mammals is shown in Fig. 1. A DNA vaccine administered to the host body can activate the host immunity in two pathways. In one pathway, the encoded antigenic protein is expressed in non-immune cells (including muscle cells and skin cells) or antigen-presenting cells (APC), such as dendritic cells (DC). The antigen peptide is presented by MHC class I or II to enhance T cell differentiation to CD4+ T cells and CD8+ CTL (Rice *et al*., 2008). The CD4+ T cells also enhance B cell differentiation to specific antibody producing cells (Coban *et al*., 2008; Stevenson *et al*., 2010). In the other pathway, DNA of the vaccine is directly recognized by some receptors including CpG DNA sensor [*i.e.* Toll-like receptor (TLR)-9], B-form DNA sensor [*i.e.* Z-DNA binding protein-1 (ZBP, also known as DAI)] and inflammasome [*i.e.* NACHT-, LRR- and pyrin domain (PYD)-containing proteins (NALP3)]. Recognition by these receptors stimulates type-I interferon (IFN) and pro-inflammatory cytokine gene expressions as an adjuvant (Coban *et al*., 2008;

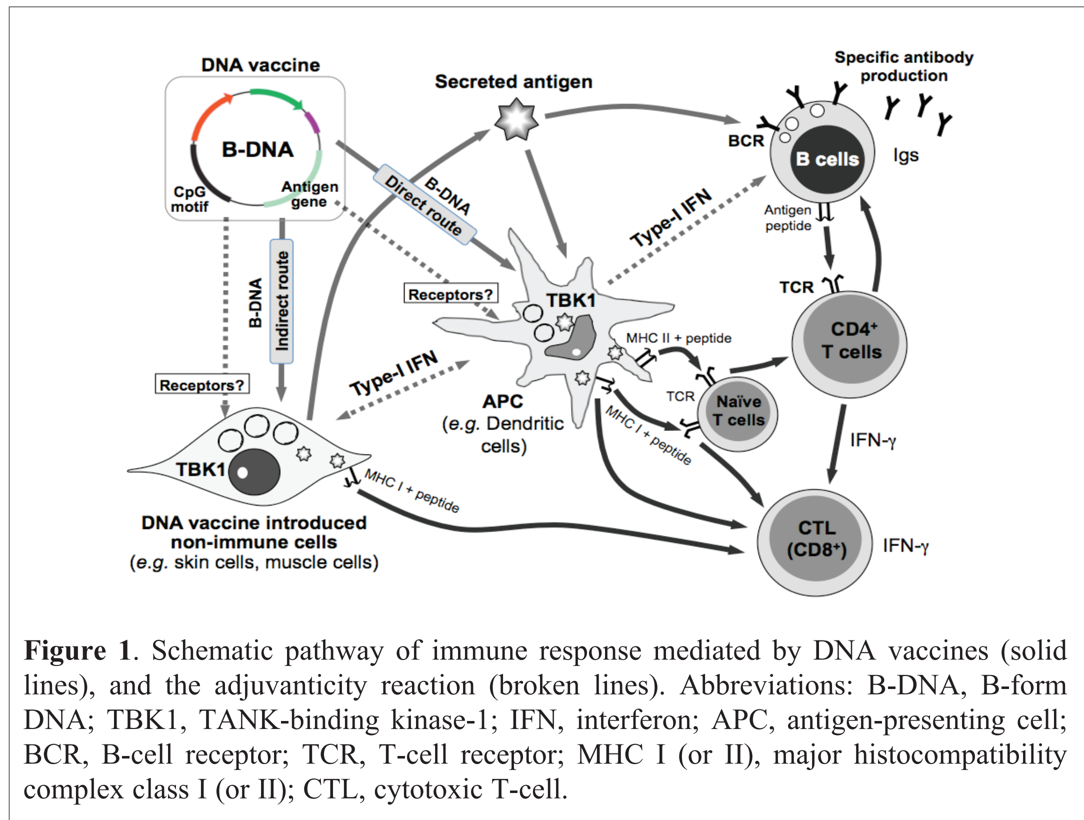

Figure 1. Schematic pathway of immune response mediated by DNA vaccines (solid lines), and the adjuvanticity reaction (broken lines). Abbreviations: B-DNA, B-form DNA; TBK1, TANK-binding kinase-1; IFN, interferon; APC, antigen-presenting cell; BCR, B-cell receptor; TCR, T-cell receptor; MHC I (or II), major histocompatibility complex class I (or II); CTL, cytotoxic T-cell.

Stevenson *et al*., 2010). In these recognition cascades, TANK-binding kinase-1 (TBK1) mediates the adjuvant effect of DNA vaccines as a key molecule, which is necessary for DNA-vaccine-induced immunogenicity (Ishii *et al*., 2008).

The microarray results showed that DNA vaccination significantly induced the expression of MHC class I transcripts (Caipang *et al*., 2006), and increased the expression of T cell activation-related genes (Yasuike *et al*., 2010). These results suggest that teleosts have an interaction pathway similar to that in mammals. Therefore, development of DNA vaccines in teleost fish will be greatly aided by understanding the cellular responses and receptors to DNA vaccines.

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Pathogen risk analysis for aquatic animals: experiences from nine case studies

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ABSTRACT

Pathogen risk analysis is an internationally accepted method for deciding whether trade in a particular commodity poses a significant risk to human, animal or plant health, and if so, what measures might be applied to reduce that risk to an acceptable level. This paper provides an overview of the qualitative risk analysis process and briefly examines the results of nine risk analyses that have been undertaken for the Asia and the Pacific Region. The risk analyses examined were conducted by Australia (3), New Zealand (4) and by the Pacific Community (2) and involved the movement of finfishes (5 cases), crustaceans (4 cases) and molluscs (1 case). Two cases involved ornamentals, five cases involved live animals to be moved for aquaculture development and two cases involved non-viable finfish product. It is concluded that although the nine case studies were all hampered by a lack of basic information on aquatic animal pathogens, they were all able to meet the three main objectives of minimizing the risk of transfer of serious pathogens and diseases between trading partners, justifying the application of sanitary measures (e.g. restrictions on species and/or sources of origin, health certification requirements, quarantine, treatment, etc.) and minimizing restrictions to trade. Past experience has shown that serious diseases are often spread through the movement of live aquatic animals and their products, which, coupled with the poor knowledge base that exists for most pathogens of aquatic animals (including information on their identities, life cycles, host specificities, geographical distributions, pathogenicities, etc.) justifies the use of precautionary approaches to minimize the risk of introducing pathogens to new hosts and geographical areas.

Key words: import risk analysis, pathogen risk analysis, risk analysis for aquatic animal movement

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INTRODUCTION

International trade continues to increase in volume due to the expanding human population and technological advancements in transport and communications. Liberalization of international trade has been in part facilitated firstly by establishment of the General Agreement on Tariffs and Trade (GATT) in 1947, followed more recently by the adoption of the *Agreement on the Application of Sanitary and Phytosanitary Measures* (the SPS Agreement) in 1994 and the creation of the World Trade Organization (WTO) in 1995.

Under the SPS Agreement, WTO Member Countries (counting 153 as of June 2008) may employ sanitary or phytosanitary measures to the extent necessary to protect human, animal and plant health. However, they must base their sanitary measures on international standards, guidelines and recommendations, which in the case of sanitary measures for aquatic animals and their products, is the World Organisation for Animal Health's (OIE) *Aquatic Animal Health Code* (OIE, 2008). WTO members may adopt higher level of standards that those specified in the Code, however, they are required to use the *risk analysis* process as a means to justify these additional restrictions on international trade (see WTO, 1994; Murray, 2002; Rodgers, 2004).

As a result, risk analysis has become recognized internationally as an appropriate method for deciding whether trade in a particular commodity poses a significant risk to human, animal or plant health, and if so, what measures can be applied to reduce that risk to an acceptable level. Besides the SPS Agreement, there are several other international treaties, agreements and memberships that affect international trade in aquatic organisms (Table 1). Some are binding agreements that involve reporting and other requirements, and some are not.

Risk analysis is usually defined either by its components and/or its processes. The Society for Risk Analysis (http://www.sera.org/) defines "risk analysis" in the following ways:

- a detailed examination including risk assessment, risk evaluation and risk management alternatives, performed to understand the nature of unwanted, negative consequences to human life, health, property or the environment;
- an analytical process to provide information regarding undesirable events; and
- the process of quantification of the probabilities and expected consequences for identified risks.

The risk analysis process has also been simply defined as "science-based decision making" (Arthur, 2008). Risk analysis has characteristics that include consistency of process, transparency of process, emphasis on stakeholder consultation, separation of the objective (scientific fact) from the subjective (opinion), emphasis on the precautionary principle, the concept of an appropriate level of protection (ALOP) (Wilson, 2001), separation of science-based and political decisions, and the concept of unacceptable risk. Risk analysis is now

widely applied in many fields that touch our daily lives. These include decisions about risks due to natural disasters, climate change, contaminants in food and water, unemployment, public security, terrorism, safety, insurance, litigation, and so on.

Table 1
Some important international and Asia-regional treaties, agreements and memberships related to international trade in aquatic organisms and their products. Adapted from Arthur *et al.* (2004)

International Law
• SPS Agreement • Convention on Biodiversity (CBD) • Cartagena Protocol on Biosafety • Convention on International Trade in Endangered Species (CITES)
International Memberships
• World Trade Organization (WTO) • World Organisation for Animal Health (OIE) • United Nations (UN) • Various regional inter-governmental associations (e.g. APEC, ASEAN, SEAMEO, SAARC, EU) [1]
Other Non-binding Codes and Agreements
• Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy (FAO/NACA, 2000) • FAO Code of Conduct for Responsible Fisheries (CCRF; FAO, 1995) • ICES Code of Practice on the Introductions and Transfers of Marine Organisms (ICES, 2005)

[1]APEC – Asia-Pacific Economic Association; ASEAN – Association of South East Asian Nations; FAO – Food and Agriculture Organization of the United Nations; ICES – International Council for the Exploration of the Sea; SEAMEO – Southeast Asian Ministers of Education Organization, SARC – South Asian Association for Regional Cooperation, EU – European Union.

RISK ANALYSIS IN RELATION TO AQUATIC ANIMAL HEALTH AND TRADE IN AQUATIC ANIMALS

In the fields of aquatic animal health and aquaculture, risk analysis has mainly been applied to assess risks to society and the environment posed by hazards created by, or associated with, aquaculture development (Bondad–Reantaso and Arthur, 2008). These include evaluating risk associated with environmental degradation, genetic impacts, introduction and spread of pests and invasive species and introduction and spread of pathogens. While the utility of risk analysis when applied to aquatic animal health is undoubtedly extensive, this paper will concentrate only on pathogen risk analysis (often termed ***import risk analysis*** when applied to international trade). Pathogen risk analysis relates to the analysis of risks of introducing and/or spreading exotic pathogens or strains of pathogens into new geographic areas with the international or domestic movement of aquatic animal commodities (i.e. live aquatic animals and their products).

The main objectives of pathogen risk analysis are:
(i) to minimize risk of transfers of serious pathogens and diseases between trading partners,
(ii) to justify application of sanitary measures (e.g. restrictions on species and/or sources of origin, health certification requirements, quarantine, treatment) and
(iii) to minimize restrictions to trade.

The *Aquatic Animal Health Code* (OIE, 2008) outlines the necessary basic steps in the risk analysis process that should be followed. However, the framework is flexible and decisions as to the details of the process are left to individual member countries.

Governments and the private sector must often make decisions based on incomplete knowledge and a high degree of uncertainty. Because of this, pathogen risk analysis is a structured process within a flexible framework within which the risks of adverse consequences resulting from a course of action can be evaluated in a systematic, science-based manner (MacDiarmid, 1997; Rodgers, 2004). The risk analysis process allows objective and transparent analysis of the risks of disease introduction associated with movements of living organisms and their products across international and domestic borders. Because of the transparent methodology, the risk analysis approach can permit a defendable decision to be reached on whether the risk posed by a particular action or hazard is acceptable or not, and provides the means to evaluate possible ways to reduce risks from unacceptable to acceptable levels.

Components of pathogen risk analysis

The main components of a pathogen risk analysis include hazard identification (i.e. What can go wrong?), risk assessment (How is it likely to go wrong and what would be the resulting consequences?), risk management (What can be done to reduce either the likelihood and/or consequences of it going wrong?) and risk communication (How do we communicate the risk to others in order to generate a change in management, regulation or operation?)

Risk analysis has only recently begun to be used widely to assess the potential risks associated with movements (and proposed movements) of aquatic animals throughout the Asia-Pacific region. Here we examine nine case studies where pathogen risk analysis was applied to movements of fish, crustaceans and mollusks in the Asia-Pacific region. Table 2 presents a summary of the main features of each study. All were based on qualitative analysis of available data, an approach often used when dealing with analyses of aquatic animals due to several factors (Table 3), not the least being the paucity of epidemiological data available, which tends to preclude use of the more involved and costly qualitative analysis method (Murray, 2002). The size and scope of the studies examined varied considerably. Six of the studies examined risks associated with proposed movements of single species (ranging from live and dead fish, to live adult and larval crustaceans, to live mollusks), while the remainder examined risks associated with movements of products from multiple species of salmonids (22 species, Stone *et al.,* 1997) and live ornamental fishes (two studies that encompassed 392

Table 2
Details of nine case studies where pathogen risk analysis was applied to proposed movements of aquatic animals in the Asia-Pacific region.

Risk assessment	Type of translocation	Number of host genera/ species considered	Number of potential hazards in preliminary list	Number of hazards fully assessed	Number of hazards requiring risk management	Risk management methods recommended or required[5]
Salmonids for human consumption (Stone *et. al.*, 1997)	International / between hemispheres	22 species	85	7[1]	0	• Pre-export certification • Commodity headed, gilled and eviscerated • Post-arrival processing at registered premises only
Live ornamental finfish to Australia (AQIS, 1999)	International / between hemispheres	605 genera	104	44	12	• Pre-export inspection and certification • Post-arrival inspection and quarantine (2-3 weeks) • Ad hoc disease testing and/or chemotherapy in quarantine • Safe disposal of wastewater and packaging
Juvenile kingfish (*Seriola* sp.) from Australia to New Zealand for seacage culture (Diggles, 2002)	International / Regional	1 species	42	9[2]	4	• Spatial separation and mortality cut off (5%) in hatchery • High salinity, no external lesions (EUS, parasites) • Virus testing programme (VER/ VNN, aquabirnavirus) • Post-arrival quarantine (4 weeks)
Postlarval blue shrimp (*Litopenaeus stylirostris*) from Brunei to Fiji (Bondad Reantaso *et al*., 2005)[4]	International / between hemispheres	1 species	21	8	8	• Larvae from specific pathogen free (SPF) broodstock • Spatial separation in hatchery • Testing for viruses (BP, HPV, IHHNV, NHP, TSV, WSSV, YHV) and *Vibrio penaeicida* • No shrimp removed from receiving facility without permit

Table 2 *(continued)*
Details of nine case studies where pathogen risk analysis was applied to proposed movements of aquatic animals in the Asia-Pacific region.

Postlarval giant river prawn (*Macrobrachium rosenbergii*) from Fiji to Cook Islands for land-based culture (Arthur *et al.*, 2005)[4]	International / Regional	1 species	61	2	2	• Virus testing programme (WSSV, MrNV, XSV) • No shrimp removed from receiving facility without permit
Ornamental fish and marine invertebrates from all countries to New Zealand (Biosecurity New Zealand, 2005)	International / between hemispheres	394 genera and species	>500	35	13	• Rationalize permitted species to remove high risk hosts • 4 to 6 weeks quarantine • Targeted passive surveillance during quarantine with mortality trigger point (20%) for disease investigation
Adult *Macrobrachium rosenbergii* from Hawaii to New Zealand for land-based culture (Biosecurity New Zealand, 2006)	International/ between hemispheres	1 species	76	6	4	• Virus testing programme (MrNV, WSSV, XSV) • Sourced from waters free of *Aphanomyces astaci* and *Angiostrongylus cantonensis* • No shrimp removed from receiving facility without permit
Menhaden (*Brevoortia* sp.) from USA to Australia for lobster bait (Diggles 2007a)	International/ between hemispheres	1 species	42	1	1	• Pre-export certification with controls on fishing locations • Virus testing (IPN-like Aquabirnavirus) • Post-arrival holding at registered premises, for licensed users only

Table 2 *(continued)*
Details of nine case studies where pathogen risk analysis was applied to proposed movements of aquatic animals in the Asia-Pacific region.

Pacific oysters (*Crassostrea gigas*) from Tasmania to New South Wales for on-growing (Diggles, 2007b)	Domestic/ interstate/ inter-island	1 species	18[3]	13	3	• Virus testing (Herpes-like virus OsHV-1) • Chlorine and/or hypoxia treatment for *Carcinus maenas* and *Undaria pinnatifida*

[1]Included in Monte Carlo Simulation Modeling.
[2]Due to lack of knowledge of kingfish diseases in Australia, this analysis considered not only diseases of kingfish but the likelihood that kingfish could be exposed to any of the notifiable diseases of finfish recorded from Australia.
[3]Includes both pests and disease agents.
[4]Analysis considered both ecological and pathogen risks.
[5] BP – *Baculovirus penaei*; EUS – epizootic ulcerative syndrome; HPV – hepatopancreatic parvo-like virus; IHHNV – infectious hypodermal and haematopoietic necrosis virus (IHHNV); IPN – infectious pancreatic necrosis; MrNV – *Macrobrachium rosenbergii* nodavirus; NHP – necrotising hepatopancreatitis; OsHV-1 – ostreid herpesvirus -1; TSV –Taura syndrome virus; VER/VNN – viral encephalopathy and retinopathy/viral nervous necrosis; WSSV – white spot syndrome virus; XSV – extra small virus; YHV – yellow head virus

Table 3
Comparison of features of quantitative and qualitative risk analysis.

Study type	Quantitative	Qualitative
Approach	Analytical	Pragmatic
Information needs	Comprehensive epidemiological data required	Data gaps acceptable
Expertise	Considerable	Experience preferred
Timeliness	Can take years	Usually months
Cost	Usually expensive	Relatively economical

(AQIS, 1999) and 605 genera (Biosecurity New Zealand, 2005). Only one study examined risks associated with domestic movements of Pacific oysters in Australia (Diggles, 2007b), with the remaining eight studies relating to international movements of fish or crustaceans.

Hazard identification

Hazard identification is the first step in the risk analysis process, and it centers around the process of identifying hazards that could potentially produce consequences. This process attempts to answer the general question "What can possibly go wrong ?" (Arthur *et al.,* 2004).

To be identified as a hazard, a pathogen typically:

- must have been reported to infect or is suspected of being capable of infecting the commodity;
- must cause significant disease outbreaks and associated losses in susceptible populations;
- could plausibly be present in the exporting country; and
- is absent from the importing country or is under an official control or eradication programme.

For the case studies examined here, there were large variations in the number of hazards identified per host species (Table 4). The "hazard:host" ratio varied from as high as 76 potential hazards per host (Biosecurity New Zealand, 2006, proposed movements of adult *Macrobrachium rosenbergii* from Hawaii to New Zealand) to as low as 0.17 hazards per host (AQIS 1999, live ornamental finfish into Australia). There was a general trend whereby the hazard to host ratio was much higher on average (43.3 hazards per host) for the six studies that examined only a single host species, while the studies that examined multiple host species considered on average only 0.67 hazards per host (Table 4). This difference was mainly due to the large numbers of species of ornamental fishes considered in two studies (AQIS, 1999; Biosecurity New Zealand, 2005), together with the paucity of information available on their diseases and pathogens (Corfield *et al.,* 2007, Whittington and Chong 2007). Even so, the risk analysis conducted on live ornamental fishes and invertebrates into New Zealand (Biosecurity New Zealand, 2005) still identified over 500 potential hazards from a host list of 394 genera and species.

Table 4
Hazards identified in the nine case studies.

Risk assessment	Number of host genera and/or species considered	Number of hazards in preliminary list	Hazard: host ratio
Salmonids for human consumption	22 species	85	3.86:1
Live ornamental finfish to Australia	605 genera and species	104	0.17:1
Juvenile kingfish from Australia to New Zealand	1 species	42	42:1
Postlarval blue shrimp from Brunei Darussalam to Fiji	1 species	21	21:1
Postlarval giant river prawn from Fiji to Cook Islands	1 species	61	61:1
Ornamental finfish and marine invertebrates to New Zealand	394 genera and species	>500	1.27:1
Adult giant river prawn from Hawaii to New Zealand	1 species	76	76:1
Menhaden from the United States of America to Australia	1 species	42	42:1
Pacific oysters from Tasmania to New South Wales	1 species	18 (includes pests)	18:1
Mean for single species risk analysis (6 studies)	1 species	43.3 hazards	43.3:1
Mean for multispecies risk analysis (3 studies)	**340 species**	**230 hazards**	**0.67:1**

Risk assessment

The next step in the risk analysis process is the risk assessment. This step is the process of evaluating the likelihood that a potential hazard will be realized, and the potential consequences of that happening. In the context of pathogen risk analysis, this usually means assessing the likelihood that a serious disease outbreak will result from the movement of a commodity over a given period of time, and estimating the likely biological, social and/or economic consequences of the introduction of that disease agent (Arthur *et al.,* 2004).

The risk assessment component of pathogen risk analysis normally consists of four subcomponents. In the release assessment, the biological pathways necessary for an importation activity to "release" (introduce) a hazard into the importing country are defined and the likelihood of that complete process occurring is estimated. Or, more simply stated, the release assessment determines the pathways that a pathogen can move with the commodity from the exporting country to the border of the importing country (Fig. 1) and the likelihood of this occurring. Similarly, exposure assessment determines the pathways by which susceptible populations in the importing country can be exposed to the pathogen and the likelihood of this occurring. Consequence assessment identifies the potential biological, environmental and socio-economic consequences expected to result from pathogen introduction, while risk estimation calculates the overall risk posed by the hazard

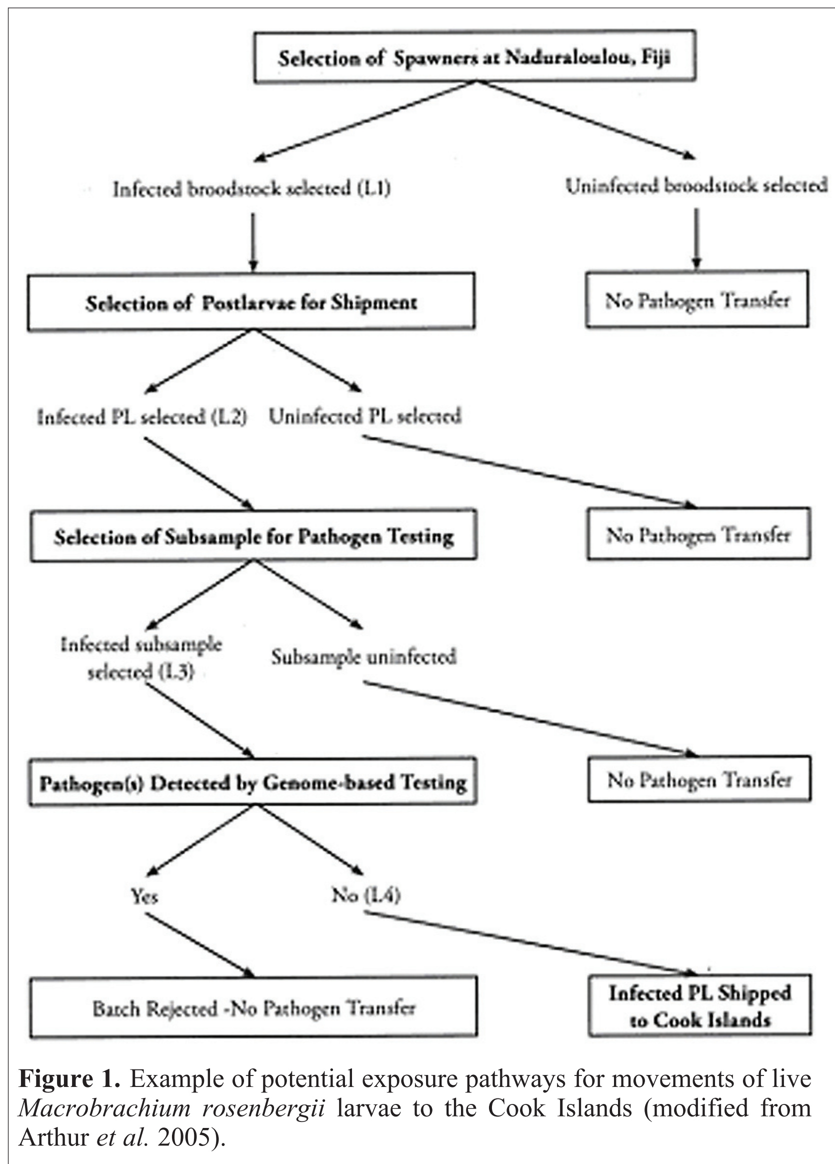

Figure 1. Example of potential exposure pathways for movements of live *Macrobrachium rosenbergii* larvae to the Cook Islands (modified from Arthur *et al.* 2005).

(the unmitigated risk) by combining the likelihood of entry and exposure with the likely consequences of establishment.

To determine whether the risk estimate for each pathogen in the risk assessment is acceptable to the importing country, the concept of a national appropriate level of protection (ALOP) is required. The ALOP (also referred to by its inverse, the "acceptable level of risk"), is the level of protection deemed appropriate by a country establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory (see WTO, 1994). As such, establishing an ALOP is a political, rather than a scientific

decision, and must be made at the highest level of government. Where no formal statement of ALOP exists, a country's ALOP can often be defined by review of its import practices for various other (often non-aquatic animal) commodities. In the risk analyses examined, determination of each country's ALOP was often demonstrated using risk estimation matrixes, which can be very useful for rapidly determining whether a country enforces a relatively low ALOP (Table 5) or a relatively high ALOP (Table 6).

Table 5
Risk estimation matrix (low ALOP/high ALOR). The shaded areas indicate situations where the unmitigated risk does not meet the country's ALOP and thus risk management will be required to reduce the risk to an acceptable level.

		Estimated Consequences				
		Negligible	**Low**	**Moderate**	**High**	**Catastrophic**
Estimated Likelihood of Release & Exposure	High	Negligible Risk	Low Risk	Moderate Risk	High Risk	Extreme Risk
	Moderate	Negligible Risk	Low Risk	Moderate Risk	High Risk	Extreme Risk
	Low	Negligible Risk	Very Low Risk	Low Risk	Moderate Risk	High Risk
	Very Low	Negligible Risk	Negligible Risk	Very Low Risk	Low Risk	Moderate Risk
	Negligible	Negligible Risk	Negligible Risk	Negligible Risk	Negligible Risk	Negligible Risk

Table 6
Risk estimation matrix (High ALOP/Low ALOR). The shaded areas indicate situations where the unmitigated risk does not meet the country's ALOP and thus risk management will be required to reduce the risk to an acceptable level.

		Estimated Consequences				
		Negligible	**Low**	**Moderate**	**High**	**Catastrophic**
Estimated Likelihood of Release & Exposure	High	Negligible Risk	Low Risk	Moderate Risk	High Risk	Extreme Risk
	Moderate	Negligible Risk	Low Risk	Moderate Risk	High Risk	Extreme Risk
	Low	Negligible Risk	Very Low Risk	Low Risk	Moderate Risk	High Risk
	Very Low	Negligible Risk	Negligible Risk	Very Low Risk	Low Risk	Moderate Risk
	Negligible	Negligible Risk	Negligible Risk	Negligible Risk	Negligible Risk	Negligible Risk

Risk management

If the country's ALOP is met, the importation can be approved without further action. However, if the risk posed by the commodity exceeds that specified by the ALOP, then additional risk management (otherwise known as risk mitigation) measures are required. For the nine case studies examined here, the risk assessment process determined that the vast majority of potential hazards did not require specific risk management. Only 8.3% of the potential hazards identified in the six single host species risk analyses and 4.6% of the potential hazards identified in the three multi-host species analyses required additional risk management measures to be implemented (Table 7).

Table 7
Results of risk assessment in the nine case studies. Note that the proportion of potential hazards that required risk management was very low.

Risk assessment	No. of hazards in preliminary list	No. of hazards assessed	No. of hazards requiring risk management
Mean for single species risk analyses (6 studies)	43.3 hazards	6.5	3.6 (8.3%)
Mean for multi-species risk analyses (3 studies)	230 hazards	26.3	10.6 (4.6%)

The option evaluation component of the risk mitigation process identifies the efficacy and feasibility of various possible measures available to reduce risks posed by the hazard. Generally, the least restrictive measure(s) found to reduce the risk to an acceptable level are selected.

During option evaluation, the risk analyst attempts to answer the question "What can be done to reduce either the likelihood or the consequences of it going wrong?" (Arthur *et al*., 2004). The process is essentially the same at that used during risk assessment, with new scenarios and pathways being constructed that incorporate steps for possible risk mitigation measures to determine their ability to reduce the overall risk (now the mitigated risk estimate) to an acceptable level.

For pathogen risk analysis, a wide variety of risk mitigation measures are potentially available to be used singly or in combination. These include pre-export health certification, quarantine (at various levels of stringency, see Arthur *et al.,* 2008), inspection, post-arrival diagnostic testing, vaccination, prophylactic treatments, use of alternate sources (e.g. specific pathogen free stocks, hatchery stocks of known health status), use of different life-cycle stages (e.g. eggs rather than juveniles or adults), or use of various treatments (e.g. cooking or other types of post-harvest processing) that reduce the risk of pathogen transfer to an acceptable level. In the nine case studies examined here, eight of the nine required pre-export disease certification or other similar conditions and seven of nine specified post-arrival quarantine or other restrictions on post-arrival movements, in no instances did the analysts determine that movements of the commodities were impossible

(Table 8). The specific risk mitigation measures recommended understandably varied on a case-by-case basis (Table 2), depending on a wide variety of commodity and country-related factors.

Table 8
Risk management recommendations from the nine case studies

Condition	Frequency of use
Pre-export certification and conditions	8 of 9 studies
Viral testing	6 of 9 studies
Post-arrival conditions/quarantine	7 of 9 studies
No movements possible	0 of 9 studies

Risk communication
One important aspect of the pathogen risk analysis procedure is risk communication, which is the process by which stakeholders are consulted, information and opinions gathered, and risk analysis results and management measures communicated. Risk communication is an essential component of any risk analysis that is conducted or commissioned by a public-sector agency (e.g. Competent Authority) and where multiple stakeholders may be involved. Such risk analyses may involve or have potential impacts upon the mandates and current and/or potential activities of a large number of agencies, organizations and individuals.

For any pathogen risk analysis, primary among the stakeholders that need to be included in communication strategies will be the proponent, the Competent Authorities in the exporting and importing countries, and the risk analysis team. However, there are usually many other entities with an interest in the outcome, the precise agencies, organizations and individuals varying depending on the commodity being considered and its intended use. Key stakeholders should be identified early in the risk analysis process and methods of advising them and seeking input established. The importance of good risk communication throughout the entire risk analysis process cannot be overstressed (Box 1).

Benefits of pathogen risk analyses
In practice, the nine pathogen risk analyses examined in this study proved to be indispensable for identifying potential disease threats, and greatly assisted with development of strategies for managing these risks, generally without undue restriction on the proposed movements of the commodities. In most cases, the risk analysis process also delivered additional benefits, such as highlighting priorities for research in those cases where data were absent or incomplete.

Although the risk analysis process is not science, it is science-based. Well qualified risk analysts are typically scientists who have considerable research experience, and high quality risk analyses utilize large amounts of supporting scientific information based on high-quality research. In many cases drafts of the analyses examined in this study were peer reviewed.

BOX 1

Risk communication case study (Biosecurity New Zealand)

Biosecurity New Zealand recently updated its risk analysis procedures (http://www.biosecurity.govt.nz/files/pests/surv-mgmt/surv/review/revision-to-risk-analysis-procedures-10oct07.pdf). Risk analyses are now released for consultation as *drafts* – with *option*(s) for different levels of intervention and related measures as opposed to *recommended* measures. Feedback will be sought from stakeholders on these options. Risk analyses will then be finalized following this consultation and will present options – refined if appropriate –for the import health standard process to consider. (Measures will only be *recommended* to the Chief Technical Officer for decision once the import health standard process is complete.) Draft and final risk analyses will be signed off by a committee chaired by the Manager of the Risk Analysis team with the Manager, International Co-ordination and Manager of Standards as the other members of the team. Decisions will be made by consensus – if this is not possible then differences will be escalated to the Director Policy and Risk and Director Standards for resolution. Decisions about the content and measures outlined in draft and final risk analyses should be guided by the Decision Making Framework – process and principles – as endorsed by the Biosecurity New Zealand Executive Management Team in March 2007 as well as our domestic and international obligations.

The scientific information used is mainly obtained from the published scientific literature, but unpublished information obtained from colleagues, as well as expert opinion was also used in some instances.

Constraints of pathogen risk analyses for aquatic animals

Of course, the risk analysis process is not perfect. The main constraints to the risk analysis process for aquatic animal pathogens in the studies examined here included:

- a lack of baseline data for hazard identification;
- scarce data on pathogen inactivation and epidemiology;
- uncertainty regarding the ecological consequences of pathogen introduction;
- uncertainty regarding the financial consequences of pathogen introduction;
- inconsistent evaluation of risk between different commodities and analysts;
- inconsistency regarding the appropriate level of protection (ALOP) between different commodities in the same country; and
- regulatory issues, especially in developing countries – (e.g. lack of disease surveillance, lack of competent authority or competent authority lacking expertise and/or diagnostic capacity in the field of aquatic animal health)

A key reason for the large amount of uncertainty that is seen during many risk analyses is the general lack of basic knowledge on the epidemiology and pathogens of aquatic animals, particularly for ornamental fishes and less commonly traded species, and especially for commodities originating from developing countries.

Are the objectives of pathogen risk analysis being met in the real world?

From the case studies reviewed here, we consider that the answer to this question is Yes, most of the time. The three main objectives of pathogen risk analysis are to minimize risk of transfers of serious pathogens and diseases between trading partners, to justify application of sanitary measures (e.g. restrictions on species and/or sources of origin, health certification requirements, quarantine, treatment) and to minimize restrictions to trade. As none of the studies examined here determined that the proposed movements of the commodities were impossible, the key criteria of minimizing restrictions to trade would appear to have been achieved. However, there have been suggestions by some authors that implementation of a precautionary approach in some instances may result in adoption of more restrictive sanitary (risk mitigation) measures that may not be justifiable in the absence of additional epidemiological information (mainly related to exposure pathways) for some commodities, such as commodity shrimp (see Flegel, 2009). However, there is also ample evidence that while the risk of transfer of serious pathogens tends to be reduced by the risk analysis process, in some cases they have not been minimized to levels consistent with the high ALOP enforced by some countries (such as Australia and New Zealand) for other commodities such as plants and terrestrial animals (Biosecurity Australia, 2009).

The precautionary principle was defined in Principle 15 of the Rio Declaration (1992) as follows:

Where there are threats of serious or irreversible environmental damage, lack of full scientific certainty should not be used as a reason for postponing measures to prevent environmental degradation.

We consider that there is sufficient evidence to warrant use of the precautionary principle during pathogen risk analysis for aquatic animals. This is particularly justifiable for hazard identification and selection of risk mitigation measures, as experience has shown that hazards can still occur even in the absence of disease identification (Gaughan, 2002). A classic example of the latter problem is the emergence of a novel herpesvirus that caused massive epizootics in Australian pilchards (*Sardinops sagax*) in 1995 and 1998 (Whittington *et al*., 1997, Hyatt *et al.,* 1997). Both epizootics extended over 7 700 km of coastline (Murray *et al.* 2003), radiating outwards from a 250 km stretch of coastline where intensive tuna ranching operations were feeding many thousands of tonnes of imported frozen baitfish (including *Sardinops* sp.) annually. Strong association of the disease outbreaks with the tuna farming process is suggested by the fact that the statistical likelihood of both epizootics randomly originating within the same 250 km stretch is 0.001 (Gaughan, 2002). Available evidence now suggests that the pilchard herpesvirus was a novel exotic pathogen that is now endemic in Australia (Whittington *et al.,* 2008), having been introduced into the country via importation of frozen baitfish for use as aquaculture feed (Gaughan, 2002).

A precautionary approach to the hazard identification process also might have prevented a potential incursion of exotic disease with proposed movements of juvenile kingfish (*Seriola lalandi*) from Australia to New Zealand (Diggles, 2002). While nodavirus

infections had never been recorded in the literature from *Seriola* spp., the precautionary approach to the hazard identification process used in that risk assessment considered that hatchery-reared juvenile kingfish may be susceptible to nodavirus strains (as well as other viruses) endemic to Australia (Diggles, 2002). Polymerase chain reaction (PCR) testing for nodavirus was subsequently included as part of the import health standard for movements of juvenile kingfish from Australia to New Zealand, and positive test results for nodavirus (Australian bass strain) were subsequently obtained from several pools of fish sampled from the proposed shipment (Crane, 2004), halting the translocation. From this, it appears that possible introduction of a pathogen previously unrecorded from New Zealand, and most significantly, also previously unrecorded from kingfish, was avoided mainly due to use of a precautionary approach to hazard identification and risk mitigation during the risk analysis process.

Further evidence that the precautionary principal is justifiable during selection of risk mitigation measures comes directly from another of the risk analyses examined in this paper (live ornamental fishes into Australia, AQIS, 1999). There is proof that despite the recommendations of that analysis, the pre-export conditions, border protection and post-arrival quarantine procedures used for live ornamental fishes in Australia remain inadequate (Whittington and Chong, 2007) and do not meet Australias ALOP (Biosecurity Australia, 2009). This has been evidenced by documentation of arrival of diseased fish into quarantine, escape of exotic pathogens from quarantine into the ornamental fish retail sector (Humphrey, 1995a, 1995b; Evans and Lester, 2001; Go *et al.,* 2005; Chong and Whittington, 2005; Go and Whittington, 2006; Corfield *et al.,* 2007) and establishment in the wild of many exotic freshwater fishes (together with their parasites and pathogens) in many parts of Australia (Humphrey and Ashburner, 1993; Lintermans, 2004; Corfield *et al.,* 2007).

CONCLUSION

Risk analysis allows for uncertainty of scientific knowledge, and for pathogen risk analyses for aquatic animals in particular, we consider that the use of the precautionary principle can be justified. This is because in most instances, critical epidemiological information is either scarce or simply not available. There are at least four points where the precautionary principle may come into play:

- during the hazard identification process;
- throughout the risk analysis process, when "cautious interim measures" are needed to ban or restrict trade until a sound risk analysis can be completed;
- during the pathways scenario portion of the risk assessment process, when sensitivity analysis may reveal key information gaps that must be addressed by targeted research; and
- during risk management, when risk mitigation measures are identified to reduce the risk to an acceptable level.

Through applying the precautionary principle, importing countries are permitted the time needed to address any important information gaps where research is needed to support sound decision-making. For the latter course of action, the risk analysis process itself also provides other important benefits, such as highlighting priorities for research in those cases where data are absent or incomplete.

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Development of the shrimp industry in the Western Indian Ocean - a holistic approach of vertical integration, from domestication and biosecurity to product certification

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ABSTRACT

The shrimp farming industry in the western Indian Ocean started with Aqualma's project in 1989, and now several companies farm shrimp in the Mozambique Channel. Despite the remoteness of these projects and their high investment and operating costs, they compete in the global marketplace by efficiently producing high value quality products. To address sustainability and biosecurity issues, Aqualma developed domesticated specific pathogen-free (SPF) broodstock of *Penaeus monodon* from western Indian Ocean stocks, which have been its exclusive source of post-larvae since 2003. Specific molecular diagnostic tools have been developed for each endemic pathogen detected since 1996, and these are used for routine surveillance of Aqualma's shrimp stocks along with histology. Biosecurity has become a major issue due to the inherent risk associated with semi-intensive farms and development of more projects in the zone. Introduction of exotic and endemic pathogens into the farms by infected wild fauna is a real concern, and reinforced biosecurity procedures are in place to mitigate this at Aqualma. Quality management through ISO 9001 helped in achieving capacity building on biosecurity. There is also a need for a national and regional level veterinary surveillance program for shrimp diseases with support from international organizations like the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE). In order to secure our investment and sustain the industry worldwide, we intend to participate in its safe development.

Key words: shrimp, Madagascar, domestication, biosecurity, certification

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INTRODUCTION

The Western Indian Ocean shrimp farming industry was developed according to an original strategy of rearing *P. monodon* in bigger semi-intensive ponds at an industrial scale. It started in Madagascar, and then extended to Mozambique and Tanzania. The Madagascan model of shrimp farming is based on rearing of *P. monodon* in earthen ponds of 5-10 ha with continuous water exchange. It is basically a combination of South American and Asian rearing techniques. It was originally developed by Aqualma, and has then become a model for other farms in the region. The targets are a maximum biomass of 1.8 to 2.2 tons / Ha at harvest, and bigger sizes at harvest depending on the clients' demand. Grow out ponds are usually stocked with juveniles of 1-2 g shrimp coming from nursery ponds (1 Ha). The industry is totally integrated vertically with synchronized supply chain, and the capacities at each production step are optimized. The production takes place throughout the year, which facilitates production planning.

It is aimed at a consistent final product of superior quality head-on shrimp mostly for European markets, positively differentiated by the consumer. The freshness of the head is essential for the product, requiring specific methods for harvest, transport and freezing to keep the texture of the flesh crunchy and salty. The costs of production are high, mostly because of difficult logistics (no roads, transports are only through boats and planes) and high-energy costs (no public network of electricity available on production sites). Therefore production performances have to be high and sustainable in order to stay economically viable.

The Aqualma farms located at Mahajamba and Besalampy have started producing since 1992 and 1999, respectively. Since then the productions are remarkably consistent despite adverse climatic events like cyclones and floods. The site selections for the farms were mainly done on otherwise unproductive salt flats with minimum mangrove clearing and the farm design promotes semi-intensive way of rearing in big ponds of 10 Ha in average. Regarding the water use, intake and discharge are separated, and the renewal is based on continuous water exchange. The feed management requires high quality feed, to ensure very high survival rates (over 75 % in pre-growth, and 90 % in grow-out in average), faster growth and lower food conversion ratio (FCR). In order to achieve higher performances, it has been necessary to stock domesticated SPF post-larvae, and master other critical steps like good pond preparation, optimized water filtration and continuous surveillance by in-house pathology laboratory. Due to the remoteness and absence of laboratories, competence has also been acquired on food safety analysis being run in an internal food bacteriology laboratory.

The social responsibility and community development activities have always been considered as part of the projects leading to building of hospitals, schools, electrification, and drinking water availability to ensure quality of life of the employees. Environmental responsibility is a major aspect of Madagascan shrimp industry, which has recently been acknowledged by several NGOs like WWF or third-party certifications. Activities

like ecological surveillance of the bay and mangrove plantation programs are routinely undertaken on each site of production. The projects conform to the recommendations made by FAO in its international principles for responsible shrimp farming (FAO/NACA/UNEP/WB/WWF, 2006), aiming a long-term sustainable business.

As a fully vertically integrated company, Aqualma has one domestication Center, with brood stock selection and nauplii production at Moramba, industrial scale post-larval production at Nosy Be and Mifoko, shrimp grow-out ponds at Mahajamba & Besalampy and related processing plants in Besakoa & Besalampy as shown in Fig. 1. It also has a feed plant in La Reunion Island and a cooking plant in Boulogne in France.

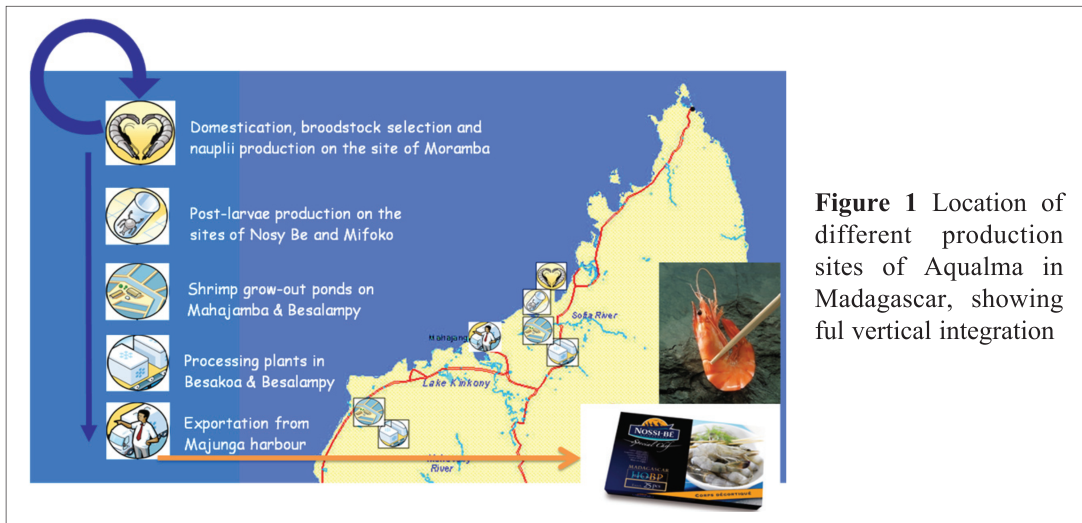

Figure 1 Location of different production sites of Aqualma in Madagascar, showing ful vertical integration

Since the species, *P. monodon*, selected for aquaculture was quite rare in Madagascan waters constituting only around 2 % of the shrimp fishery (Fig. 2), Aqualma decided to start a domestication project in order to be sustainable on a long term, though several previous attempts concluded that *P. monodon* was difficult to domesticate and produce on a commercial scale (Pullin, Williams and Preston, 1998).

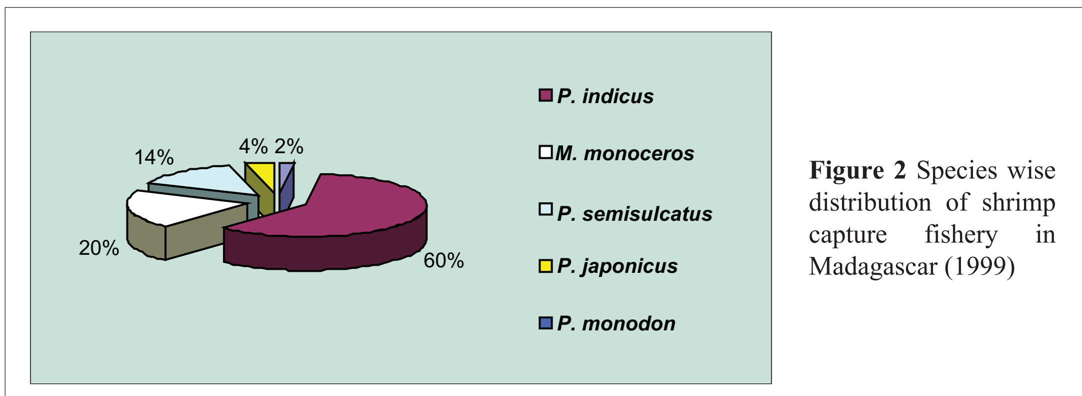

Figure 2 Species wise distribution of shrimp capture fishery in Madagascar (1999)

This paper presents a successful holistic approach in developing a sustainable shrimp farming industry involving various components. After a short description on how domestication program and wild broodstock selection was initiated, the importance of identification of endemic pathogens and development of adequate diagnostic tools for the sustainability of semi-intensive rearing method will be stressed out, leading to biosecurity management at different levels, from compartmentalization at a company level to global sanitary policy at National or even regional levels.

Other essential aspects ensuring success include optimizing rearing conditions and husbandry practices to ensure higher levels of animal welfare and adequate management of genetic resource, which will be further commented.

Finally, the achievement of complete traceability along the supply chain and product certifications to ensure maximum food safety and service to the customer will be described.

Initiation of the domestication program

The following were the objectives and expectations of the company from its domestication program:

- Independence from wild stocks and year round guaranteed supply.
- More consistent performances than wild stocks, allowing more precise projections and budget calculations. The aim was to obtain performances at least equal to wild brood stock (percentage of spawning females, eggs & nauplii per female, quality of nauplii, survival rate in larval rearing) with an expected improvement through domestication and natural selection after several generations.
- Providing a safe source of specific pathogen-free (SPF) seeds, certified free of all economically important pathogens (eventually to all viruses and intracellular bacteria, which is much easier to achieve in domesticated stocks), as it can be checked over several generations.
- Providing a base for further work on selective breeding of important traits like growth, resistance to stress or to a specific disease (Specific Pathogen Resistant).
- Providing a potential to develop more intensive, biosecure systems due to an enhanced control of disease.
- Opening an economical potential for brood stock / seed stock export business.

Maximizing the genetic diversity and better quarantine were considered as key elements in the initial stage of domestication. The founding populations of *P. monodon* were all endemic to Madagascar and collected in deep water from all the major fishing zones of the island, as presented in Fig. 3 along the western coast of Madagascar in order to start with a maximum genetic diversity. Particular attention was made to avoid those animals being captured in shallow water along the coasts to limit the risks of contamination of pathogens from other species of crustaceans. All incoming brood stocks were submitted to a primary quarantine; wherein a complete evaluation of sanitary status was performed through molecular biology and histology methods was done for all OIE listed shrimp pathogens in addition to local

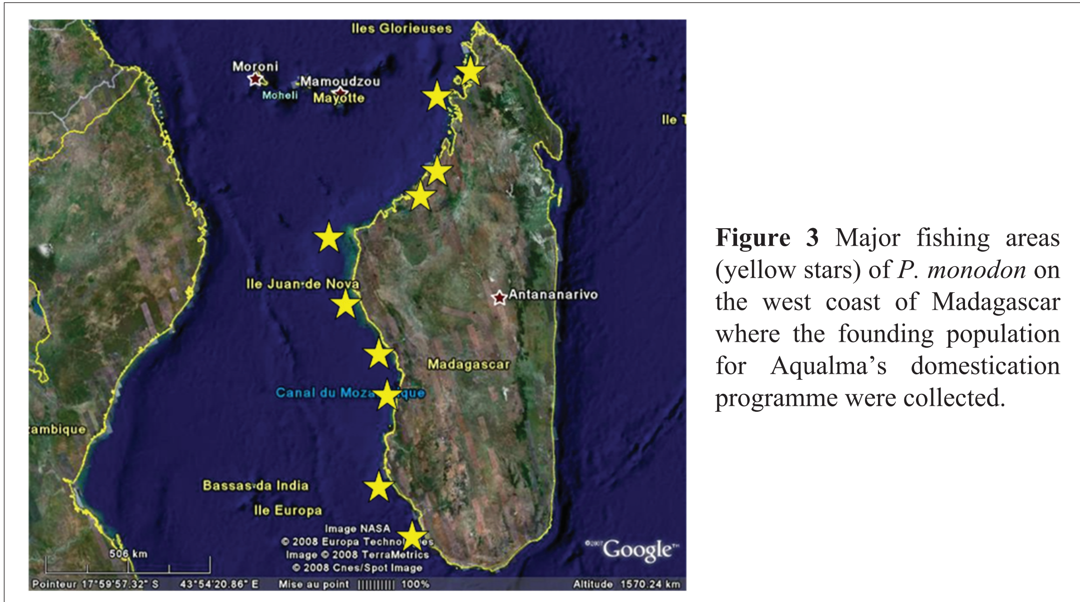

Figure 3 Major fishing areas (yellow stars) of *P. monodon* on the west coast of Madagascar where the founding population for Aqualma's domestication programme were collected.

pathogens known at the time. During this pre-domestication phase, adaptation of wild animals to the culture environment and the continuous controlled reproduction are being considered as major issues (Bilio, 2007). Once they cleared quarantine, they were used for reproduction, and their offspring reared in batches. Systematic sanitary control was performed on each generation, for each batch produced, at each growing step (Fig. 4). Runts, animals with clinical signs or abnormal behavior were scrutinized, fixed and studied, and each suspicious batch was eliminated. The absence of all major endemic pathogens discovered / detected in Madagascar since 1999 until 2008 was ensured, progressively demonstrating an SPF status for this population.

Figure 4 Facilities involved in closing the biological cycle of *Penaeus monodon* in the Domestication Centre of Aqualma, site of Moramba

The final expected product of domestication is an SPF breeder with good growth and high fecundity, genetically defined through its pedigree and physically identified through individual tagging. Based on the post-larvae requirements of the farms, simulations are made approximately 18 months before on the number of brood stocks to be produced, in order to be able to supply the required amount of post larvae on time for stocking the ponds. The performance of first generations of domesticated breeders was variable, but stabilized to satisfactory levels due to improvements in animal husbandry and selection. They exhibited subsequently good fertility and fecundity rates, producing an average of over 400,000 nauplii per spawn, similar to what can be produced from wild females of the same weight. This demonstrated that the animals adapted well to its controlled environment, and that the chosen rearing methods were adequate.

In terms of impact of domestication on shrimp production of the company, the increasing availability of this domesticated SPF population since the year 2000, provided a safe and consistent source of seeds to Aqualma, thus playing a major role in the sustainability of its results, and allowed it to pursue its development strategy. The estimated quantity produced by Aqualma with a domesticated SPF broodstock is over 30,000 tons as of January 1st, 2007, compared to 48,000 tons of total shrimp production since the beginning in 1992. The part of domesticated animals reared progressively increased from 10% to 100 % of the total post-larvae produced from the year 2000 until 2003, and has been kept 100 % since then (Fig. 5).

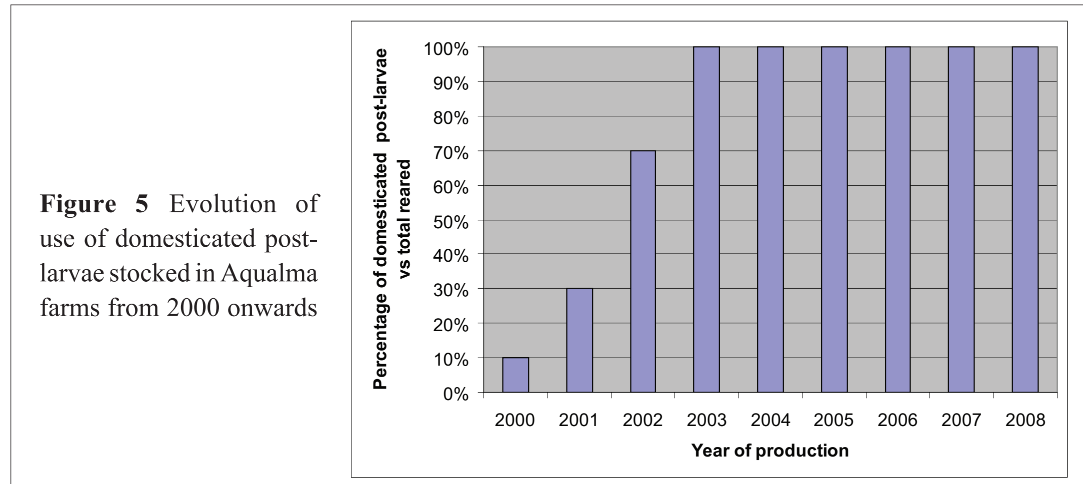

Figure 5 Evolution of use of domesticated post-larvae stocked in Aqualma farms from 2000 onwards

Acquiring knowledge on local pathogens

At the start of the project, there was little or no information available on the sanitary status of wild populations of shrimp and other crustaceans. Consequently the company took every effort to rapidly acquire and continuously update knowledge on endemic pathogens of crustaceans that may impact its operation through pathogen surveillance programs coordinated with the group company's fishing boats. Absence of competent local scientific institutions on this domain forced the company to work with international institutions like

the Shrimp Pathology Laboratory, University of Arizona, with which we signed a yearly renewed Technical Agreement, and occasionally with several other research institutes like University of Montpellier II, University Blaise Pascal-Clermont II and others. Collaboration with these institutes resulted in in-house capacity building in terms of developing diagnostic facilities (histopathology and molecular diagnostics), training of manpower and routine counter analysis. Thanks to these collaborations and its internal team, the company was able to participate actively in new discoveries of endemic pathogens (Fig. 6) and in the development of specific diagnostic tools. As Madagascar still continues to enjoy pathogen-free status with respect to many pandemic pathogens like WSSV, YHV, TSV, Brazilian IMNV and NHP, histopathology is still being considered as a principal diagnostic tool as the majority of diseases cases reported were due to local unknown pathogens, or non OIE listed pathogens. A well-equipped molecular biology laboratory with PCR and *in situ* hybridisation capabilities together with histology laboratory form an essential component in strategies to assure bio-security to various production units. Following are the endemic pathogens that were identified during this period of time, and taken into account in the surveillance program.

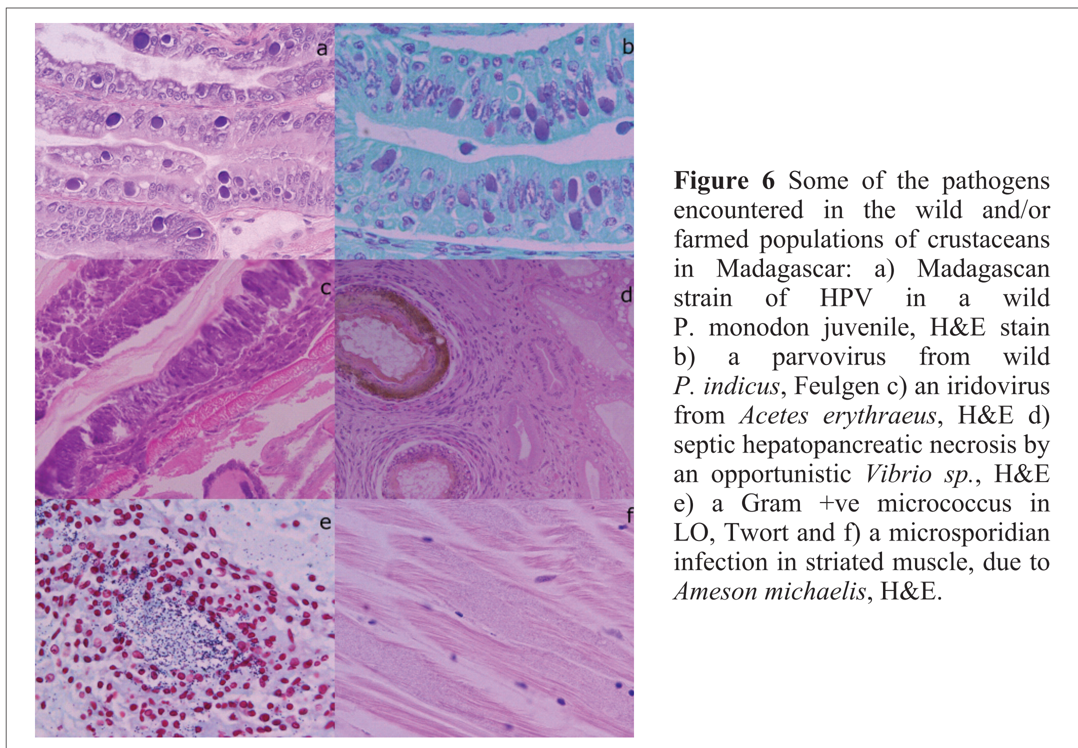

Figure 6 Some of the pathogens encountered in the wild and/or farmed populations of crustaceans in Madagascar: a) Madagascan strain of HPV in a wild P. monodon juvenile, H&E stain b) a parvovirus from wild *P. indicus*, Feulgen c) an iridovirus from *Acetes erythraeus*, H&E d) septic hepatopancreatic necrosis by an opportunistic *Vibrio sp.*, H&E e) a Gram +ve micrococcus in LO, Twort and f) a microsporidian infection in striated muscle, due to *Ameson michaelis*, H&E.

Rickettsia-like bacterium

A rickettsia-like bacterium (RLB) was reported in 1999 to be responsible for severe mortalities of farmed *P. monodon* in the southwest region of Madagascar. Specific molecular methods, PCR and *in situ* hybridization assays, were developed (Fig. 7) for these rickettsia

(Nunan *et al.*, 2003; Nunan *et al.,* 2003). So far, it has been considered to be transmitted horizontally through wild crustacean vectors like crabs, and vertical transmission has never been observed (Le Groumellec, M. and Duraisamy P., pers. comm.).

Iridovirus

An iridovirus (tentatively named SIV, sergestid iridovirus) was reported to cause high mortality in a sergestid shrimp, *Acetes erythraeus* in 2004. However there was no mortality observed in *P. monodon* population. A PCR method and *in situ* hybridization assays were developed anyway, thus allowing monitoring the evolution of this pathogen in case it becomes a threat in the future (Tang *et al*., 2007).

IHHNV

As for as IHHNV is concerned, neither symptoms nor lesions were observed in the massive monitoring programme spanning over 10 years. The published information (Tang *et al.,* 2003; Krabsetsve, Cullen and Owens, 2004) purportedly involving IHHNV strain from Madagascar might have been due to the false positive PCR reaction obtained with *P. monodon* from the region that are known to have an IHHNV related sequence with a high degree of similarity inserted in their genome. Development of a discriminating PCR assay is now available to distinguish real IHHNV from virus-related sequences in the genome of *P monodon* (Tang, Navarro and Lightner, 2007).

Hepatopancreatic Parvovirus

A strain of HPV is reported with high prevalence (Tang, Pantoja and Lightner, 2008) in the wild shrimps and found to be different from HPV isolates from Tanzania, Korea, Australia and Thailand based on nucleotide sequence analysis. However, this strain of HPV seems to never have been reported to cause mortality or have any negative impact in the farms that continue to depend on brood stock from the wild under the rearing condition adopted by Madagascar farmers (Le Groumellec, M. and Duraisamy P., pers. comm.).

Microsporidians

Presence of at least two species of microsporidians was reported in the wild population of penaied shrimp, *Fenneropenaeus indicus*, *P. monodon* and *Penaeus semisulcatus*, in the west coast of Madagascar (Toubiana *et al.,* 2004). Strain specific molecular diagnostic tools (PCR primers and *in situ* hybridisation probes) were developed to monitor potential carriers like several types of wild crustaceans.

Monodon Slow Growth Syndrome, local form (MSGS)

Detection of a previously undescribed virus in the lymphoid organ and gills of growth retarded population of *P. monodon* from a commercial shrimp farm has been reported in the zone, suggesting resemblance with monodon slow growth syndrome (MSGS) that has been problematic with *P. monodon* in Thailand since 2001 (Anantasomboon *et al*., 2006). Efforts are being made to understand more about the condition and to develop a diagnostic

tool, as this syndrome could be of major concern for the industry if its presence in the zone is confirmed. Meanwhile histology diagnostic remains adequate to demonstrate the absence of such a syndrome in our domesticated stocks or farms.

Others pathogens

Wild populations of *P. monodon* are sporadically observed with MBV, but seem to have never impacted the production (Le Groumellec, M. and Duraisamy P., pers. comm.). A baculovirus was reported from crab. Similarly a parvovirus infecting the midgut caecum was observed in the wild populations of F. indicus. These findings were made thanks to the pathogen surveillance program, performed originally by Aqualma internal pathology laboratory until 2007, now extended to the whole Madagascar island and integrated with the national surveillance program. Infection by opportunistic bacterial pathogens like common Vibrio spp. infections, other specific Vibrio inducing systemic infections like *Vibrio nigripulchritudo* and a Gram+ micrococcus have also been reported.

Considering all these newly discovered pathogens, a biosecurity policy was first designed at a company level, with the future intention to amplify it to a national level.

Biosecurity strategies – company, country and regional levels

At a company level, compartmentalisation of production systems was already considered at the design stage (Fig. 7). Compartments separate animals at different phases of the production cycle with distinct health status and comfort levels (Zepeda, Jones, and Zagmutt, 2008). Biosecurity measures to prevent introduction of infections from one compartment to the others is assured by implementing control plans that are part of the standard operating

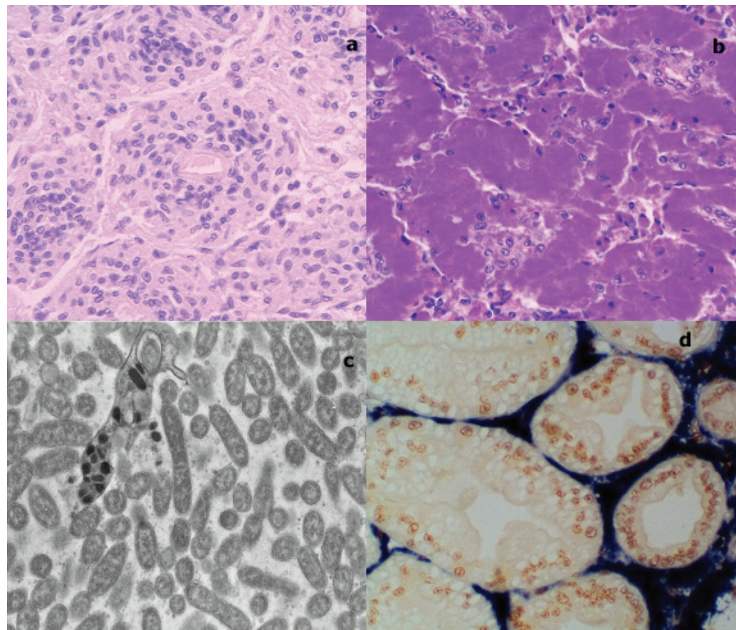

Figure 7 Different steps leading to development of molecular diagnostic tools for endemic pathogens of Madagascar, i.e. for RLB. a) Normal lymphoid organ (LO) tubules, H&E stain b) LO tubules infected with RLB, H&E c) TEM image of RLB in LO and d) positive reaction to *in situ* RLB probe in the hepatopancreas, showing the systemic infection, not affecting the tubules. Photos c & d are from Shrimp Pathology Laboratory, UAZ.

procedure of each compartment. A crisis management procedure is in place to react quickly to disease outbreaks in any of the compartments. Equipments, training of personnel, predefined responsibilities and preparedness are essential component of this crisis management system. Each compartment is audited by independent agencies for certifications (ISO 9001 V 2000; Label Rouge; ACC).

As the domestication centre and larval rearing facility are closed units with more indoor facilities, the production of post larva from domesticated brood stock is done in a more secured environment guaranteeing constant production of SPF seeds for stocking at the farms. All the other inputs used in the hatchery production are sourced from certified suppliers and each lot of inputs undergoes rigorous quality control before being used in the system, and complete traceability records are maintained. Quarantine facilities, filtration and sterilisation of both inlet and outlet water, systematic screening for pathogens at different life stages and control over movement of workers and materials are part of the biosecurity plan.

The grow out farms are semi-closed systems where there is total control of animal movements, but limited control over water flow. They are vulnerable to natural calamities like cyclone and flooding resulting in stress for shrimp and significant changes in the wild fauna (Fig. 8). A continuous monitoring of environmental parameters and surveillance programme is used to manage any potential biosecurity issues arising out of these natural events.

Figure 8 View of typical semi-intensive farms in Madagascar; a) Mahajamba Farm (700 Ha, 3200 tons average per year), Aqualma and b) Besalampy farm (450 Ha, 2000 tons average per year), Aqualma.

Each production site has a dedicated person in charge of biosecurity and animal husbandry issues, who works in coordination with the company's veterinary doctor and central laboratory. They are also in charge of monitoring the wild fauna and potential vector populations in the surrounding environment including the bays. Based on risk analysis, methods are developed to prevent their contact with the reared animals in the ponds. Periodic review of the process helps in constantly improving the biosecurity protocols, critical control points, surveillance programs, contingency plans, emergency harvests and secured disposal and elimination procedures.

The other major issue for Madagascar producers was to keep the status of the island free from the major OIE listed pathogens affecting the shrimp industry worldwide. As the farming industry in western Indian Ocean really started after the devastating WSSV outbreaks in Asia, the industry in the region had sufficient lessons to learn from others mistakes, and thus diseases were perceived as one of the major issues threatening sustainability. Consequently, substantial efforts and investments were made in projects like Aqualma ranging from domestication to separation of various production units to prevent the entry of pathogens and to limit the spread of any potential disease outbreaks. A law was enforced in 2003, prohibiting all entrance of live crustaceans, and fixing rules regarding the rearing conditions and animal welfare in the farms.

By virtue of isolation from other shrimp farming countries and being an island, Madagascar is free from most of the OIE listed pathogens. At country level, Madagascar is eligible to make self-declaration of disease-free zone status based on historical data and a starting epidemiology surveillance program. The Association of shrimp farming and fishing companies in Madagascar (Groupement des Aquaculteurs et Pêcheurs de Crevettes de Madagascar - GAPCM) and the competent authority (Autorité Sanitaire Halieutique - ASH) have been promoting this strategy since several years, through the raising of funds for the newly setup epidemio-surveillance national aquatic pathology laboratory and organizing seminars with an international epidemiology expert to validate the original survey plan and follow-up (Cameron, A., pers. comm.) in order to reach this goal that will benefit to all the operators in the country. To maintain the disease-free status, effective mechanisms in terms of legislation to prevent spread of diseases through trans-boundary movements of aquatic animals needs to be enforced. Adoption of codes of practice, adherence to industry standards set up in association with international groups i.e., WWF and exigencies of clients are some of the ways to assure bio security at national level, which involves the competent authority (Autorité Sanitaire Halieutique - ASH), the concerned Ministry (Ministère des Pêches et des resources Halieutiques) and the Association of shrimp farming and fishing companies in Madagascar (GAPCM) and has a clear regulatory frame (Government of Madagascar, 1990, 2001, 2001; Bis, 2004).

The region still continues to be free of pandemic pathogens that are known to cause devastating epidemics in shrimp farms. As the countries around the Mozambique Channel are far from other major shrimp farming regions of the world, a regional setup similar to the Network of Aquaculture Centres in Asia and the Pacific (NACA) is needed to coordinate with world bodies like OIE and FAO on various issues concerning aquatic animal diseases. The company, deeply conscious of the need to have a common strategy to ensure the biosecurity of this zone, is strongly promoting this regional discussion.

Optimization of rearing conditions and animal husbandry

Once the foundation for domestication of *P. monodon* was established, the objective was then to start the second phase of the domestication process, mostly by designing sustainable and optimized animal breeding techniques. We developed exhaustive rearing protocols that

are adapted to our requirements throughout many assays. As this form part of the intellectual property of Unima/Aqualma group, the details cannot be divulged, but the general method used will however be presented, as it is not yet very common in shrimp aquaculture.

In terms of management requirements, the most important aspect of this domestication project is to have a strong discipline on documenting what has been done or tested. All the performances should be recorded in databases in order to analyse and apply a continuous improvement to provide the best conditions to the breeders, respecting animal welfare as far as possible. Working with a tool for quality management also helps, as the interaction of all these aspects is critical (Fig. 9). We propose a modified version of Sniezko's diagram, more adapted in our opinion to its use in private industry. It is very important to distinguish what is controllable from what is not in the environment of the shrimp. All actions performed by the farmer which can reduce stress and improve animal welfare should be identified and put in place. The overall success of all these issues also largely depends on manpower management. We chose to work under ISO 9001:2000 certifications, with written procedures, records on critical points and continuous improvement. All the production steps are audited and certified by a third-party annually. Specific training procedures adapted to local culture were also designed. Under semi-intensive rearing conditions, in ponds without aeration, we obtained satisfactory results (Fig. 10) thanks to a dedicated farm management. Typical performance figures obtained are an FCR of 1,41 for a mean weight at harvest of 30g, with a survival rate of 86 % (stocking weight at 1,52 g).

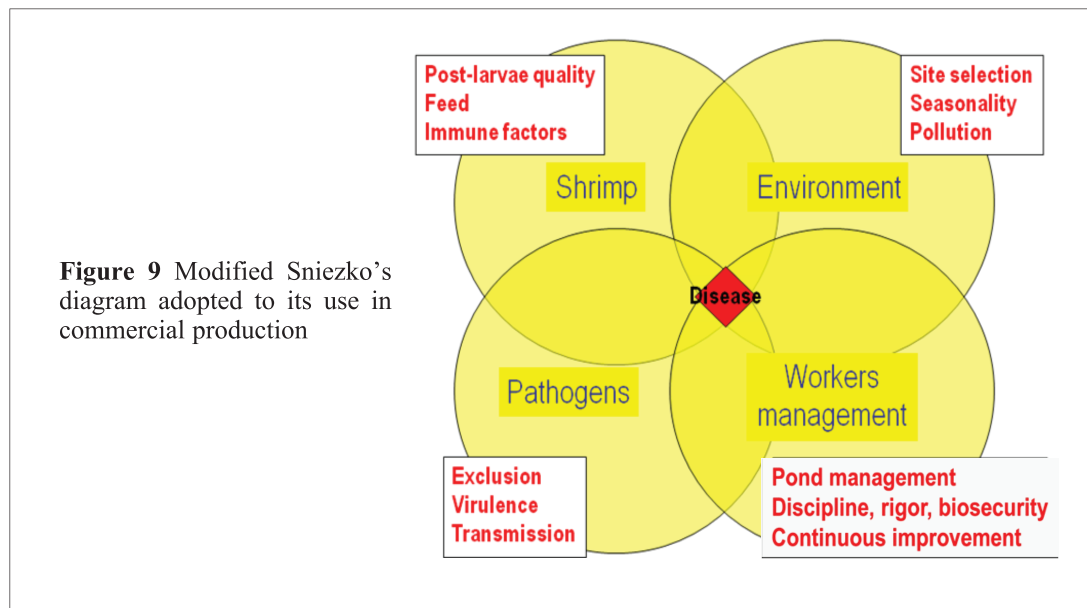

Figure 9 Modified Sniezko's diagram adopted to its use in commercial production

Genetic resource management

The main purpose was to maximize the genetic diversity of the founding population. In order to obtain this diversity, as mentioned elsewhere, the breeders were collected from several locations of Madagascar Island. Considering the number of breeders collected and

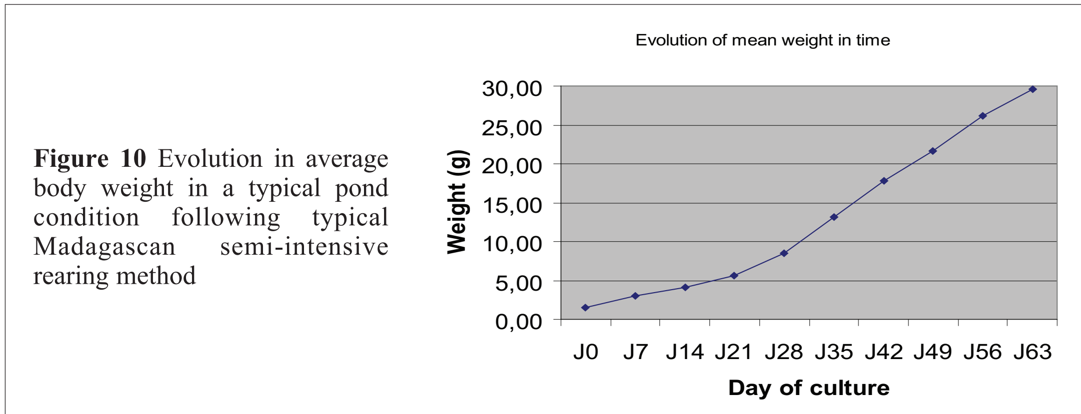

Figure 10 Evolution in average body weight in a typical pond condition following typical Madagascan semi-intensive rearing method

selected, the domesticated population was initiated from 198 wild founders, representing more than 99.5% of the genetic variability in the wild population. They were selected from approximately 10,000 breeders, notably according to their sanitary status and their performances.

Moreover, crossing optimization was performed from the beginning in order to minimize inbreeding. Since 2003, the domestication centre functions only with this genetically optimized population: 140 families are reproduced per each generation, divided in 7 sub-cohorts with a rotational mating scheme. The effective population size is maintained over 300, allowing an excellent control of inbreeding, which increases less than 0.2% at each generation, which ensures the sustainability of the genetic management program. The family traceability is maintained over the generation via visible implant elastomer (Northwest Marine Technology, USA). A mild within-family selection for growth is applied to eliminate potential runts.

Figure 11 shows the evolution of growth rate with generations of domestication in *P. monodon*. The growth rate plotted here is the growth from 2 to 30 g in small outdoor ponds, corrected for variations in rearing temperature, and then gives an unbiased estimate of the trend of growth rate (15% per generation of domestication). However, this trend is not corrected for environmental effects other than temperature (i.e. improvements in husbandry techniques, feed, rearing density etc.) and therefore cannot be considered as an unbiased estimate of the genetic gain from domestication, but only as an upper limit of such an estimate.

The future of this genetic program is expected to be based not only on quantitative genetics, but also by using molecular tools to trace parentage (suppression of the separate rearing phase before tagging). It is an undergoing work in collaboration with a research team. Mass selection can also be performed, notably to better evaluate the heritability of growth in this population and for rapid improvement of this trait. In the long term, disease resistance and fertility improvement could also be included in the breeding goal.

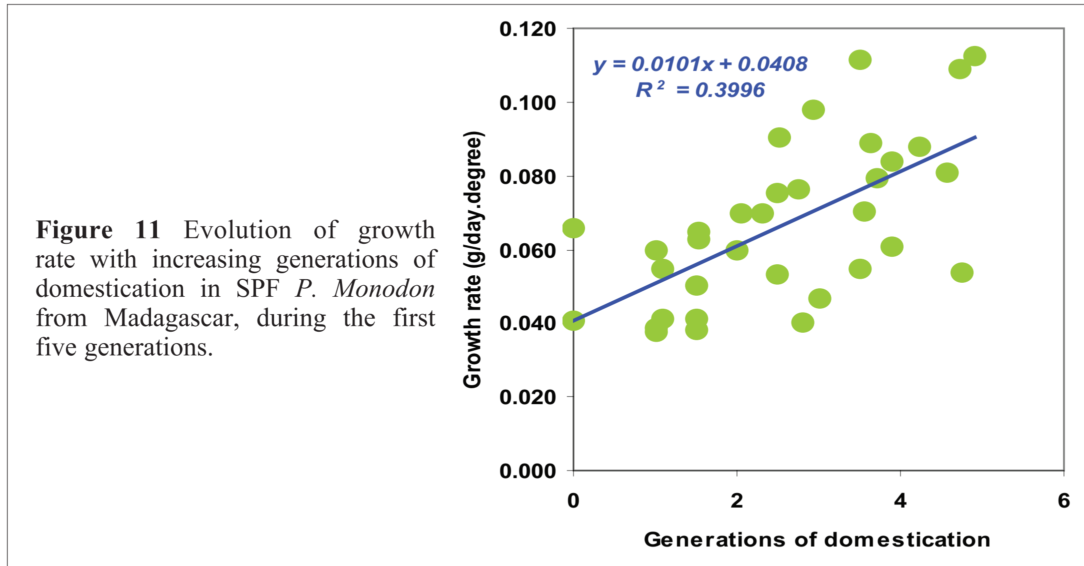

Figure 11 Evolution of growth rate with increasing generations of domestication in SPF *P. Monodon* from Madagascar, during the first five generations.

Building databases and traceability records

Once the computer databases were built, we developed interfaces for different users, including the technicians, biologists, economists, logisticians and managers. Portability of these different databases through links results in obtaining complete traceability from founding populations to finished products including all the inputs used in the production process. Complete traceability is necessary to address our clients' enquiries about the causes of incidents, and therefore call back exercises are performed frequently. Software has been developed to help the sellers to extract information from the databases and to be able to track every batch produced.

Process and product certifications

In order to evidence these achievements and to be able to value those commercially, it has been decided to initiate product certifications.

Decision was to stress out the quality of the finished product through a famous standard called "Label Rouge", where the quality and its consistency are asserted by blind tests performed by third party. Along with very strict and detailed standards regarding the rearing methods and continuous control procedures, it assures that the consumer will consistently get a superior quality product. This required an involvement of all supply chain actors, as the certification covers all steps of production, from broodstock management to marketing, thus requiring a holistic and integrated approach.

In parallel, efforts made on respecting high standards regarding environmental and social issues were distinguished by a close partnership with the World Wildlife Fund.

In the recent years, our production sites were thus audited and certified by Aquaculture Certification Council (ACC), and continued to be for Label Rouge, WWF and several food safety standards like IFS and BRC.

CONCLUSION

We successfully domesticated *P. monodon* from Madagascar 10 years ago, and produced over 30,000 tons of superior quality head-on shrimp for European markets since then. The domesticated SPF shrimp population allowed us to produce consistently despite our open semi-intensive model that is highly susceptible to environmental changes, using better biosecurity and management control. This was notably achieved through the extensive use of ISO 9001:2000. Being fully integrated vertically allows us to provide complete traceability, therefore to obtain famous certifications, hence our products carry multiple labels like the “Label Rouge” in France, which was completed by the development of a private partnership with WWF to communicate on Aqualma’s environmental and social respectful management. The genetic management program is sustainable, providing animals with good performance and future selection programs look promising. The involvement of the private sector - along with other stakeholders - in research and development, and its importance in leading biosecurity measures can also be stressed out from our experience, when initiating a shrimp industry in an new country.

We are also looking at opportunities to share experience and acquired know how with partners who have common values of long term sustainable business for mutual benefits. One objective is to assess the potential of this domesticated stock to perform well under different rearing conditions (notably the current Asian rearing model).

We used domestication program and biosecurity as elements of a sustainable strategy, by reducing impacts of shrimp culture on its environment (no catches of wild breeders, no disease dissemination). The shrimp industry has been responsible so far for the worldwide spread of many crustacean pathogens. It can be viewed as a failure, and shows that we must be pro-active and promote alternative strategies.

The domestication is required for all species in aquaculture in order to sustain at industrial level. This also helps to control the sanitary level of the reared populations, to stabilize, to optimize and then to improve their rearing performance through selective breeding. This should be done while respecting the basics of genetics to limit inbreeding. Each domestication process should be adapted to the farmers’ will and needs.

Like with terrestrial animals in the past, aquaculture species should soon be fully domesticated, specific pathogen free and genetically managed in closed conditions. We plead for the development of local domestication programs, allowing each region to promote its own strains and own breeding methods, leading to the emergence of several “races” for each species reared (shrimp, crabs, etc.). In order to achieve this, we will need a strong multi-disciplinary effort through all stakeholders and researchers (physiology, physiopathology),

and this should be pro-actively promoted by international organizations like FAO and OIE as major actors in regional integration strategies and providers of trainings for capacity building, for crustacean aquaculture to become really sustainable.

Moreover, the biggest danger of shrimp aquaculture in the near future is in our opinion what is currently happening with domesticated *Litopenaeus vannamei*, as it leads to worldwide uniformity and ease of production. Pressure from the market in terms of price induces drop in quality, and consequently a change in the mind of the consumer, now seeing shrimp as a commodity product. This has already caused serious economical problems to the farmers, especially in less developed countries, unable to compete with these falling prices. Product diversity, quality of finished products, eco-friendly and sustainability driven certifications are some of the alternatives to this disastrous trend, and we intend to promote this alternative strategy of development for crustacean aquaculture.

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Minimum inhibitory concentrations of antimicrobials against clinical *Vibrio* and *Streptococcus* isolated from aquaculture

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ABSTRACT

Four antimicrobial agents: Amoxycillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) and Sulfadimethoxine/Ormetoprim (ORS) were tested for their *in vitro* antimicrobial activity against clinical *Streptococcus* and *Vibrio* isolates from clinical cases occurring between 2005 to 2006. The specimens or moribund animals from different culture areas in Thailand were collected for laboratory microbiological analysis. *Streptococcus* strains were isolated from the kidney of the diseased tilapia *Oreochromis niloticus* and *Vibrio* strains were obtained from the hepatopancreas of diseased black tiger shrimp *Penaeus monodon* or pacific white shrimp *Litopeneaus vannamei*. All clinical bacterial isolates were identified by conventional tube media or API system (Biomerieux, France). The agar dilution method, as described by the Clinical and Laboratory Standards Institute (CLSI), was used to determine Minimum Inhibitory Concentrations (MICs) of each chemical against the tested isolates. The effect of the components of seawater on the antimicrobial activity of ORS was also analysed by a comparison between MIC values tested on Mueller Hinton Agar (MHA) dissolved in distilled water with an added 1% NaCl and on agar dissolved in 10 ppt seawater. MIC values suggested that *Streptococcus* isolates obtained from the diseased tilapia were resistant to Oxytetracycline (MIC range 0.50-8.00 µg/ml) but susceptible to Amoxicillin (MIC range 0.031-0.250 µg/ml), STX (MIC range 0.285/0.015-9.50/0.50 µg/ml) and ORS (MIC range 0.152/0.008-4.75/0.25 µg/ml). While the test conducted against *Vibrio* isolates revealed broader MIC ranges; Amoxicillin 1.0-512 µg/ml, Oxytetracycline 1.0-512 µg/ml, STX 1.178/0.062- >152/8 µg/ml and ORS 0.152/0 008- >152/8 µg/ml, the activity of ORS was not substantially influenced by an addition of 10 ppt seawater to the tested system. .The study concluded that, in respect of MIC testing and the possible implications of a seawater effect, ORS is a functional antimicrobial for the tilapia *Streptococcus* and penaeid shrimp *Vibrio* pathogen.

Maisak, H., Tipmongkolsilp, N. and Wongtavatchai, J. 2011. Minimum inhibitory concentrations of antimicrobials against clinical *Vibrio* and *Streptococcus* isolated from aquaculture, pp. 309-316. *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.

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Keywords: minimum inhibitory concentrations, antimicrobials, *Streptococcus spp.*, *Vibrio spp.*

INTRODUCTION

Bacterial diseases are considered to be the most important cause of mass mortality and economic loss when present in intensive aquaculture. Antimicrobial therapy is one of the strategies frequently applied to control bacterial diseases. As part of the prudent use of animal antimicrobials, antimicrobial susceptibility testing is strongly recommended prior to treatment. The aim of this study was to determine the Minimum Inhibitory Concentrations (MICs) for 4 antimicrobial agents: Amoxycillin, Oxytetracycline, Sulphadiazine/Trimethoprim (SXT) and Sulfadimethoxine/Ormetoprim (ORS) against *Vibrio* isolated from diseased Black Tiger shrimp (*Penaeus monodon*) and Pacific White shrimp (*Penaeus vannamei*), and *Streptococcus* isolated from diseased tilapia (*Oreochromis nilotica*) in Thailand. The MIC tests reveal the antimicrobial susceptibility of bacterial pathogens associated with the aquaculture industry of Thailand and hence, provide data to direct the prudent use of antimicrobials in aquaculture production.

MATERIALS AND METHODS

Bacterial Strains

Test strain: Fifty four *Streptococcus* spp. isolates were derived from tilapia from disease cases occurring between 2005 and 2006. Streptococcosis has been observed in most regions of tilapia farming; in the north-eastern, eastern and central parts of Thailand. Isolates were identified by conventional biochemical methods described in the API system (BioMerieux, Marcy l'Etoile, France). Of the fifty four *Streptococcus* strains isolated from the kidney of the diseased tilapias were 4 *S. dys. equisimilis*, 43 *S. agalactiae*, 6 *S. porcinus* and one *Streptococcus* sp. All bacterial strains were stored in a maintenance broth containing 40% glycerol and supplemented with 10% fetal bovine serum, at -70 ^{O}C. Before each experiment the stored bacteria strains were transferred to Tryptic Soy Agar (TSA, Difco Laboratory, USA) supplemented with 10% sheep blood. After incubation at 30 ^{O}C for 18-24 hr, the inocula were transferred to Tryptic Soy Broth and the cell density was adjusted to McFarland standard 0.5 or approximately 10^8 Colony Forming Unit (CFU)/ml. The inocula were then diluted ten-fold in sterile normal saline, giving a final cell density of approximately 10^7 CFU/ml.

Vibrio isolates derived from clinical cases of diseased penaeid shrimp were obtained from the culture collection of the Department of Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The collection has culture specimens from disease cases occurring between 2004 and 2006, mainly from the western and southern parts of Thailand. Previously, the identification of the isolates had been performed using conventional biochemical methods described in the API system (BioMerieux, France). Of the fifty *Vibrio* strains isolated from the hepatopancreas of the diseased penaeid shrimp were

4 *V. alginolyticus*, 7 *V. cholerae*, 7 *V. damsela*, 14 *V. fluvialis*, 10 *V. parahaemolyticus* and 8 *V. vulnificus* strains. All bacteria strains were stored in a maintenance broth containing 40% glycerol, at -70 ^{O}C. The isolates were treated with similar procedures described for streptococcal isolates, but 1%NaCl was added to Tryptic Soy Agar.

Quality control strains: Additional bacterial organisms were obtained from the American Type Culture Collection for use as quality controls: *Esteriachia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *V. parahaemolyticus* ATCC 17802.

Antibacterial Agents

The antimicrobials that were tested were Amoxicillin, Oxytetracycline, Sulfadiazine, Trimethoprim (Sigma Chemical Co., USA.), Sulfadimethoxine and Ormetoprim (PHARMAQ, St Louis, MO, Norway). A serial two-fold dilution of antimicrobials dissolved in solvent was processed with distilled water, except that Trimethoprim and Ormetoprim were dissolved in solvent only as suggested by CLSI, giving a series of tested concentrations.

Minimum Inhibitory Concentration (MIC)

The procedures described here are in accordance with the international recommendations provided by the Clinical and Laboratory Standards Institute (CLSI). Mueller Hinton Agar (MHA) plates, containing serial two dilutions with the four antimicrobial agents, were inoculated with a standardized inoculum of the test strain (10^7 CFU/ml). Using a standard multipoint inoculator, bacteria from mature cell cultures were allocated at approximately 10^4 CFU/spot on the surface of MHA. After 18-20 hr., incubation, the MIC was recorded as the lowest concentration of antimicrobials with no visible growth of bacteria. The minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolate were reported as MIC_{50} and MIC_{90}, respectively. Additional tests on Meuller Hinton Agar, dissolved in sea water (10 ppt salinity), were performed to evaluate any possible effects of components in the sea water that might affect the antivibrio activity of Sulfadimethoxine and Ormetoprim. Quality controls of the methods were regularly performed on each test. Inhibition of the quality control strains by antimicrobial agents are required to be comparable with the interpretive standards.

RESULTS AND DISCUSSION

The values of the MICs of Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) and Sulfadiamethoxine/Ormetoprim (ORS) against 54 *Streptococcus* isolates associated with the diseased tilapia are presented in Table 1. The MIC_{50} and MIC_{90} were, respectively, the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates. The data showed that Amoxicillin, SXT and ORS were effective against the tested *Streptococcus* isolates and their MIC_{90} corresponded with the susceptible range of MIC interpretive standards against *S. pneumonia* (CLSI, 2002) (Table 2).

The distribution of MIC values also correlated with the estimated values of MIC_{50}, MIC_{90} and the MIC range, showing that ORS had a better overall MIC range than did other antimicrobials (Fig. 1).

Table 1
Minimum Inhibitory Concentrations (MICs) of antimicrobials against 54 *Streptococcus* isolates associated with tilapia disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
Amoxicillin	0.062	0.125	0.031-0.250
Oxytetracycline	0.500	8.000	0.500-8.000
Sulfadiazine/Trimethoprim (19:1)	2.375/0.125	4.750/0.25	0.285/0.015 – 9.50/0.50
Sulfadimethoxine/Ormetoprim (19:1)	2.375/0.125	4.750/0.25	0.15/0.008 – 4.750/0.25

Table 2
MIC Interpretive Standards (µg/ml) for S. pneumoniae using the Agar Dilution Method (CLSI, 2002)

Antimicrobials	MIC Interpretive Standard (µg/ml)		
	Susceptible	Intermediate	Resistance
Amoxicillin	≤ 0.5	1	≥ 2
Tetracycline	≤ 2	4	≥ 8
Sulfamethoxazole/Trimethoprim	≤9.5/0.5	19/1-38/2	≥76/4

MIC testing for 50 *Vibrio* isolates associated with the diseased black tiger shrimp or Pacific white shrimp showed MIC_{50}, MIC_{90} and a MIC range in Table 3. MIC values of antimicrobials observed in the study, in comparison to MIC interpretive standards (Table 4), indicated that most of *Vibrio* isolates were susceptible to ORS and moderately susceptible to SXT and Oxytetracycline, while being resistant to Amoxicillin. The MIC distribution figures also showed that the frequency of MICs in the susceptible range were observed more in ORS than other compounds (Fig. 2).

Changes in MIC values due to the addition of seawater to the medium have been reported for several antimicrobial agents, resulting in implications for MIC testing (Lunestad and Samuelsen, 2001). In the present study, moderate differences in the MIC values of ORS were noticed between *Vibrio* strains tested on MHA dissolved in distilled water, with 1% NaCl added and MHA dissolved in seawater. The test on MHA dissolved in distilled water, with an added 1% NaCl, showed a MIC_{50} and MIC_{90} of 1.178/0.062 (Sulfadiamethodoxine/Ormetoprim) µg/ml while the MICs observed in a test on MHA dissolved in seawater was 1.178/0.062 (Sulfadiamethodoxine/Ormetoprim) µg/ml for MIC_{50}, and 4.750/0.250 (Sulfadiamethodoxine/Ormetoprim) µg/ml for MIC_{90} (Table 5).

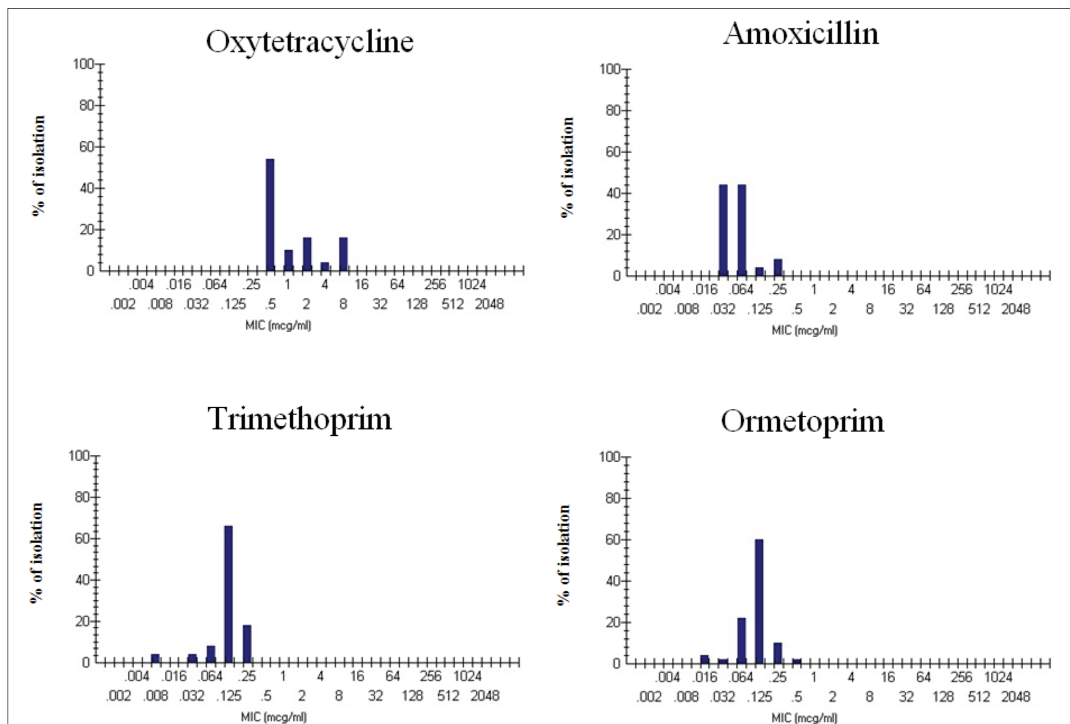

Figure 1. Frequencies of the Minimum Inhibitory Concentrations (MICs) observed for four Antimicrobials; Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) (19:1) and Sulfadimethoxine/Ormetoprim (ORS) (19:1); against 54 *Streptococcus* isolates associated with tilapia disease.

Table 3
Minimum Inhibitory Concentrations (MICs) of antimicrobials against 50 *Vibrio* isolates associated with penaeid shrimp disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
Amoxicillin	64	512	1-512
Oxytetracycline	2	64	1-512
Sulfadiazine/Trimethoprim (19:1)	9.500/0.500	>152/8	1.178/0.062 – >152/8
Sulfadimethoxine/Ormetoprim (19:1)	1.178/0.062	152/8	0.152/0.008 – >152/8

The frequencies of MICs observed for ORS indicated that the distribution of isolates with different MIC values was within the susceptible range and comparable in both test conditions, distilled water and seawater (Fig. 3). Our observation that ORS did not show a significant increase in MIC values when tested on a seawater based medium, compared to a 1% NaCl supplemented medium, is necessary for the optimal therapeutic application of the compound, particularly when the compound is used in a marine environment.

Table 4
MIC Interpretive Standards (μg/ml) for Vibrio cholerae using the Agar Dilution Method (CLSI, 2002).

Antimicrobials	MIC Interpretive Standard (μg/ml)		
	Susceptible	Intermediate	Resistance
Amoxicillin	≤ 8	16	≥ 32
Tetracycline	≤ 4	8	≥ 16
Sulfamethoxazole/Trimethoprim	≤ 38/2	-	≥ 76/4

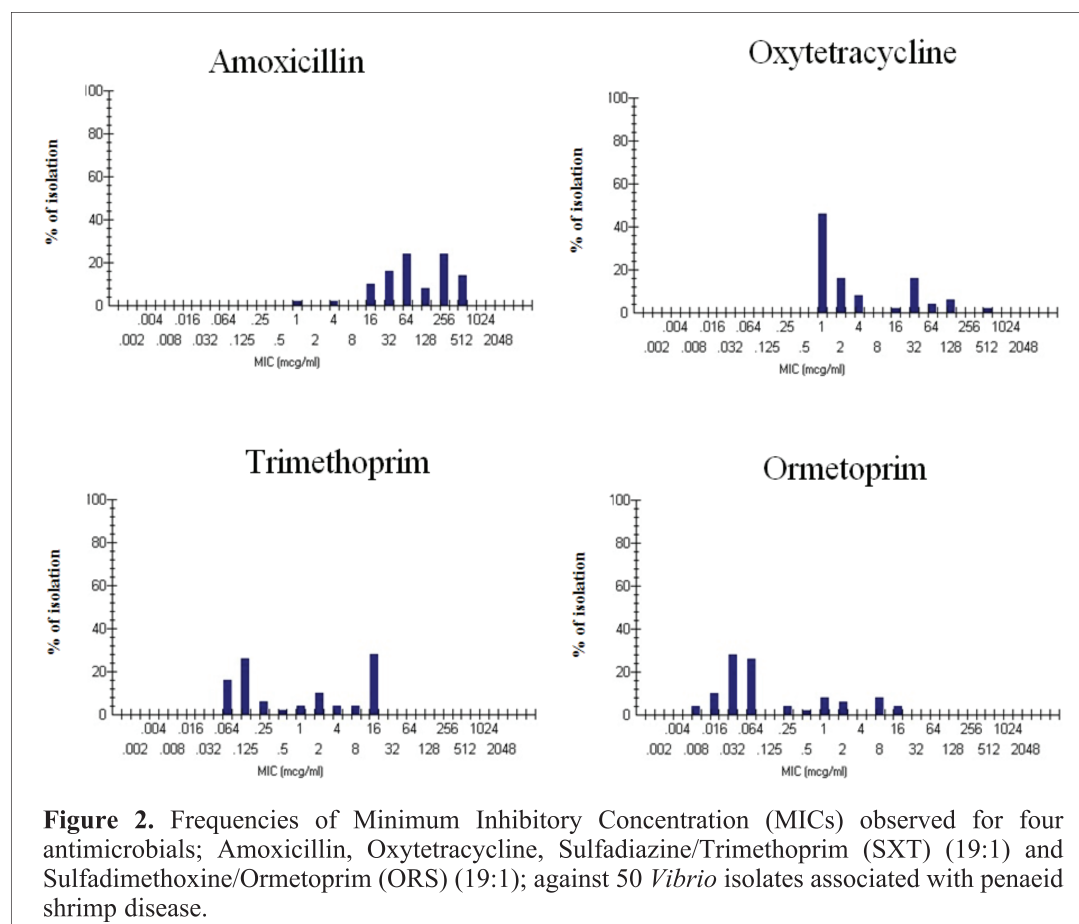

Figure 2. Frequencies of Minimum Inhibitory Concentration (MICs) observed for four antimicrobials; Amoxicillin, Oxytetracycline, Sulfadiazine/Trimethoprim (SXT) (19:1) and Sulfadimethoxine/Ormetoprim (ORS) (19:1); against 50 *Vibrio* isolates associated with penaeid shrimp disease.

Additional tests were conducted for SXT and ORS to compare the MIC testing system using different ratios of Sulfadiazine/Trimethoprim and Sulfadiamethoxine/Ormetoprim. The agar dilution method described by CLSI indicates the ratio to be used in the MIC testing of Sulfadiazine to Trimethoprim is 19:1, however, the ratio commonly used in veterinary formulae of these compounds is 5:1. Therefore, the present study compares MIC values of SXT and ORS of both ratios. The antimicrobial activity of SXT or ORS against 22 *Streptococcus* isolates, considering the MIC of Trimethoprim or Ormetoprim, were the same for both ratios (19:1 and 5:1) (Fig. 4). Corresponding results were also observed in MICs

Table 5
Minimum Inhibitory Concentrations (MICs) observed for Sulfadimethoxine/ Ormetoprim (19:1) that was tested on Meuller Hinton Agar dissolved in distilled water (DW) and in 10 ppt seawater (SW), against 22 *Vibrio* isolates associated with penaeid shrimp disease. MIC_{50} and MIC_{90} were the minimum concentrations of antimicrobials required to inhibit 50% and 90% of the tested isolates.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
	Sulfadimethoxine/Ormetoprim		
DW	1.178/0.062	1.178/0.062	0.152/0.008 – 19/1
SW	1.178/0.062	4.750/0.250	0.589/0.031 – 19/1

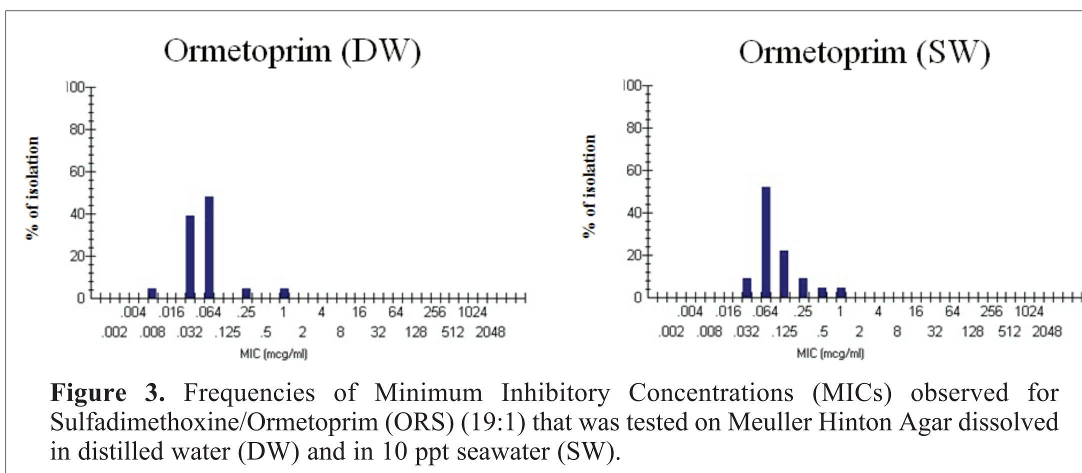

Figure 3. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Sulfadimethoxine/Ormetoprim (ORS) (19:1) that was tested on Meuller Hinton Agar dissolved in distilled water (DW) and in 10 ppt seawater (SW).

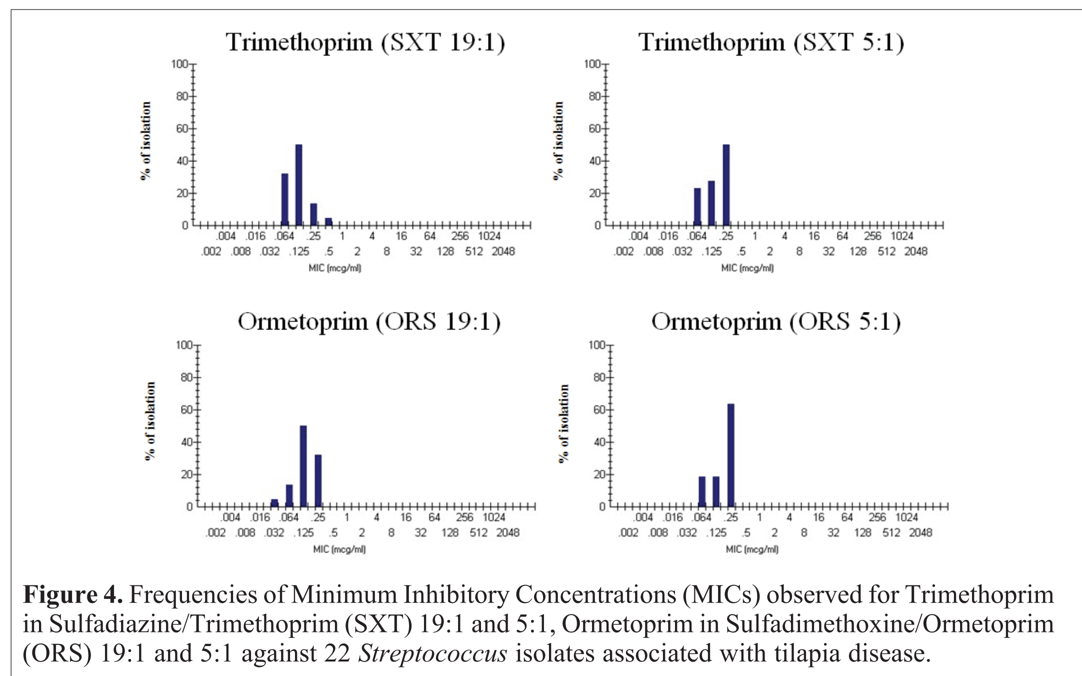

Figure 4. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Trimethoprim in Sulfadiazine/Trimethoprim (SXT) 19:1 and 5:1, Ormetoprim in Sulfadimethoxine/Ormetoprim (ORS) 19:1 and 5:1 against 22 *Streptococcus* isolates associated with tilapia disease.

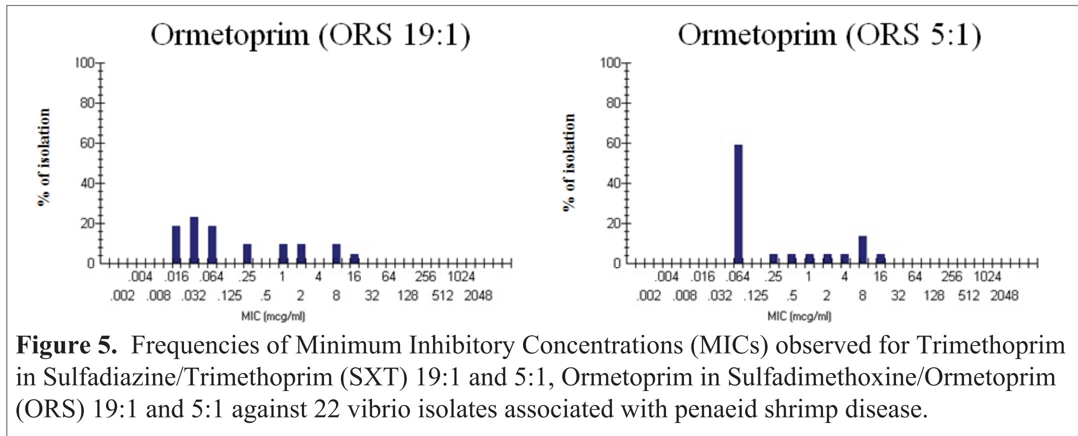

Figure 5. Frequencies of Minimum Inhibitory Concentrations (MICs) observed for Trimethoprim in Sulfadiazine/Trimethoprim (SXT) 19:1 and 5:1, Ormetoprim in Sulfadimethoxine/Ormetoprim (ORS) 19:1 and 5:1 against 22 vibrio isolates associated with penaeid shrimp disease.

tested against 22 *Vibrio* isolates (Fig. 5). Consequently, the MIC values of SXT and ORS acquired from the CLSI described ratio of 19:1 were used for the study's interpretation.

In conclusion, MIC data obtained in this study suggests that *Streptococcus* spp. isolated from diseased tilapia and *Vibrio* spp. from diseased penaeid shrimp are susceptible to ORS and SXT. Seawater has a minimal influence on the antimicrobial activity of ORS and this compound is approved for use in food fish species of many countries, making it the preferred therapeutic for aquaculture.

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Studies on the development of a vaccine against *Mycobacterium* sp.

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ABSTRACT

Mycobacteriosis, caused by *Mycobacterium* sp., results in severe loss of fish production in Japan's aquaculture industry. Since the bacterium can survive and grow in side host cells, chemotherapeutic agents are not effective and potent vaccines are required to control the disease. In this study, the effects of two vaccine candidates, Bacillus Calmette and Guèrin (BCG, an attenuated strain of *Mycobacterium bovis*) and formalin-killed cells (FKC) of *Mycobacterium* sp. strain 012971, on the immediate and acquired immune response were evaluated in Japanese flounder, *Paralichthys olivaceus.* On 1, 3 and 7 days post treatments with these candidates, gene expression levels of inflammatory cytokines encoding interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNFα) increased. This suggests that the vaccine candidates stimulate immediate immune responses. To determine if specific defenses against mycobacteriosis were acquired, purified protein derivative (PPD) from *Mycobacterium* sp. strain 012971 was injected intramuscularly into fish at 4 weeks after vaccine treatments and the inflammatory cytokine gene expression levels were analyzed. Gene expression levels of the inflammatory cytokines were increased only for the fish treated with BCG, but not those with FKC. Subsequently, the vaccinated fishes were challenged with *Mycobacterium* sp. The relative percentage survival (RPS) of BCG and FKC vaccinated groups at 12 days post challenge were 37.5 and 18.1, respectively. These data indicate that BCG might be useful as a vaccine against fish mycobacteriosis.

Key words: mycobacteriosis, live attenuated vaccine, BCG, innate immunity, cell-mediated immunity

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INTRODUCTION

Mycobacterium species are gram positive, aerobic, acid-fast, non-motile bacteria that cause mycobacteriosis in various fish species. Three species of *Mycobacterium* that are major pathogens of fish are *M. marinum* (Aronson, 1926), *M. fortuitum* (Ross and Brancato, 1959) and *M. chelonae* (Bruno *et al*., 1998). However, mycobacteriosis also has been attributed to other *Mycobacterium* species and synonyms for the three major species (Decostere, Hermans and Haesebrouck, 2004).

In 1985, an epidemic caused by *Mycobacterium* sp. affected cultured yellowtail (*Seriola quinqueradiata*) in Sukumo bay, Japan (Kusuda, Kawakami and Kawai, 1987). The symptoms of the disease in yellowtail were haemorrhagic ascites, hypertrophy of the spleen and kidney with tubercles and visceral adhesions (Kusuda, Kawakami and Kawai, 1987). Later, the disease was also shown to affect striped jack (*Pseudogaranx dentex*) (Kusuda *et al.*, 1993) and amberjack (*Seriola dumerili*) and has, to date, resulted in severe loss of fish production in Japan's aquaculture industry. The species name of the bacteria has not been identified yet, although biochemical characteristic had been examined (Kusuda, Kawakami and Kawai, 1987).

Pathogenic mycobacteria are capable of intracellular parasitism. The bacteria can avoid initial degradation in the phagosome by producing superoxide dismutase that eliminates oxygen radicals (Yamamoto, 2006). Furthermore, they can survive inside macrophages by secreting the enzyme protein kinase G out of the phagosome, assisting in the prevention of phagosome-lysosome fusion (Nguyen and Pieters, 2005). The infected macrophages are enveloped by immune cells to avoid dissemination of the bacteria, resulting in the formation of tubercules or granuloma formation in the host.

The effects of chemotherapeutic agents are limited *in vivo*, because mycobacteria can survive within host macrophages. In fact, although rifampicin, streptomycin and erythromycin can inhibit the growth of *Mycobacterium* sp., the bacteria can survive in the fish and then proliferate after the antibiotic treatment has ceased (Kawakami and Kusuda, 1990). Therefore, it is important to develop a potent vaccine against the disease.

In order to control mycobacteriosis, a potent vaccine is required to induce cell-mediated immunity in the host. *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) is a live attenuated vaccine developed from the *M. bovis* strain almost a century ago, and is still commonly used worldwide. Besides its efficacy against human tuberculosis caused by *M. tuberculosis*, BCG is also known to provide protection against leprosy caused by *M. leprae* (Ponnighaus *et al.*, 1992) and Buruli ulcer caused by *M. ulcerans* (Portaels *et al.*, 2002, Portaels *et al.*, 2004 and Nackers *et al.*, 2006) in human. Live attenuated vaccine causes chronic, but weak infection in the host. Hence, these vaccines can confer specific cell-mediated immunity and long protective efficacy. Nevertheless, the exact mechanisms of BCG-induced protection in human and animal models are still unclear (Sander and McShane, 2007).

The tuberculin response, a delayed-type hypersensitivity (DTH) response, is used to test whether the host has previously been exposed to mycobacterial antigens, including BCG vaccination. Intradermal injection of mycobacterial purified protein derivative (PPD) as an antigen elicits hall mark responses including indurations, swelling and monocytic infiltration into the site of the lesion within 24 to 72 hours (Black *et al*., 1999). Upon injection of the antigen, Langerhans cells process and present the antigen to the local memory T cells. These T cells secrete numerous cytokines and chemokines including interleukin-1 (IL-1), IL-6, IL-8, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) (Grabbe and Schwarz., 1998 and Black *et al.*, 1999) that result in vasodilatation, an influx of immune cells and activation of macrophages. As a result, granulomatous inflammation occurs at the site of PPD injection, ceasing only after degradation of the antigen.

The innate immune response, the first line of defense against pathogenic microbes, plays a critical role during mycobacterial infection. Toll-like receptor 2 (TLR2) which exists on the surface of the phagocyte recognizes pathogen-associated molecular patterns (PAMPs) of mycobacteria and activates MyD88, resulting in the induction of inflammatory cytokines (Underhill *et al.*, 1999). The activation of the innate immune response mediated by TLRs is a vital step for triggering acquired immunity. For example, TLR2 signaling leads to the release of an instructive cytokine, such as IL-12 which drives the differentiation of naïve T cells to Th1 cells (Akira, Takeda and Kaisho, 2001). Therefore, their ligands such as mycobacterial cell wall, lipopolysaccharide (LPS) and CpG-rich DNA are often used as vaccine adjuvants.

In order to develop an effective vaccine against *Mycobacterium* sp. infection, two vaccine candidates, BCG and the formalin-killed cells of *Mycobacterium* sp. strain 012971 were tested in Japanese flounder (*Paralichthys olivaceus*) as a model. Fish treated with the candidates were evaluated by quantification of the inflammatory cytokine gene expression levels during the immediate immune response and DTH response. Furthermore, protection efficacy of the candidates was confirmed by intramuscular challenge with live *Mycobacterium* sp.

MATERIALS AND METHODS

Fish rearing and bacteria propagation

Japanese flounder (*Paralichthys olivaceus*) weighing approximately 10 g were kept in 60 L tank with circulation systems maintained at 20 ºC. They were fed every other day until use.

Mycobacterium sp. strain 012971, which was isolated from cultured yellowtail (*Seriola quinqueradiata*), was grown on 1 % Ogawa medium (Nissui, Japan) at 25 ºC for 3 weeks. *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) was grown on 1 % Ogawa medium at 37 ºC for 3 weeks.

Vaccine preparation

To prepare the live attenuated vaccine, *M. bovis* BCG was suspended in sterilized phosphate buffered saline (PBS) using a glass homogenizer. One hundred microliters of the bacterial suspension was spread on Middlebrook 7H10 agar (Becton, Dickinson and Company, USA) supplemented with OADC Enrichment (Becton, Dickinson and Company, USA) and cultured at 25 ºC for 4 weeks to calculate the number of colony forming units (CFU). For the preparation of formalin-killed cells of *Mycobacterium* sp. as an inactivated vaccine, the bacteria was suspended in 10 ml of PBS containing 5 % formaldehyde (Wako, Japan) and incubated at 25 ºC for 48 h. Cell count for *Mycobacterium* sp. was done as described above. To confirm the complete inactivation of the bacteria, 100 µl of suspension was spread on Middlebrook 7H10B agar with OADC Enrichment and incubated at 25 ºC for 4 weeks.

Immediate immune response after vaccination

BCG (1.2×10^8 CFU/fish) or FKC (2.0×10^8 CFU/fish) suspended in PBS were intramuscularly injected into Japanese flounder. The kidneys of the vaccinated fish (n=3) were collected at 1, 3 and 7 days post-vaccination. Total RNAs were extracted from the kidney using RNA iso (TAKARA, Japan), following manufacturer's instructions. RNA concentration was determined by Gene Quant (Pharmacia, USA) and stored in DEPC treated water at -80 ºC until used.

First strand cDNA was synthesized with 2 µg of total RNA using MMLV reverse transcriptase (Invitrogen, USA), following manufacturer's instruction. The changes in mRNA levels for Japanese flounder inflammatory cytokines including IL-1β (GenBank Accession number AB070835), IL-6 (DQ267937) and TNFα (AB040448) in fish at 1, 3 and 7 day post-vaccination were determined by quantitative RT-PCR (qPCR). Primers for qPCR were designed using Primer Express Software Version 3.0 (Applied Biosystems, USA) including ribosomal protein L10 (RPL10, AU050650) used as internal control (Table 1). The reaction mixture contained 5 µl of the diluted cDNA sample, 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 0.4 µl of the forward and

Table 1
Primers used in this study.

Primer	Sequence
IL-1β short F	5'-CAGCACATCAGAGCAAGACAACA-3'
IL-1β short R	5'-TGGTAGCACCGGGCATTCT-3'
IL-6 short F	5'-CAGCTGCTGCAAGACATGGA-3'
IL-6 short R	5'-GATGTTGTGCGCCGTCATC-3'
TNF α short F	5'-CGAAGGCCTAGCATTCACTCA-3'
TNF α short R	5'-TCGTGGGATGATGATGTGGTT-3'
RPL10 short F	5'-GCTCCTCTGGTGCAGTTTGTGA-3'
RPL10 short R	5'-TGGTGTTTGCTGGCGTCACTCT-3'

reverse primer (10 μM) designed for each gene were adjusted up to 20 μl with sterilized water. qPCR was performed using 7300 Real Time PCR System (Applied Biosystems, USA), according to manufacturer's instruction.

Preparation of purified protein derivative (PPD)

The purified protein derivative (PPD) was prepared from *Mycobacterium* sp. strain 012971. The bacteria were cultured in 200 ml of Middlebrook 7H9 broth (Becton, Dickinson and Company, USA) supplemented with OADC Enrichment for 8 weeks at 25 ºC. After heat sterilization at 105 ºC for 3 h, bacterial cells were removed by centrifugation at 200,000×g for 1 h and the supernatant was passed through Syringe Driven Filter Unit (Millipore, USA). The filtrate was then concentrated by boiling for 30 min and this was followed by the addition of 40% (w/v) trichloroethanoic acid (Wako, Japan). The precipitate was collected by centrifugation at 6,500×g for 15 min. After washing with 4% (w/v) trichloroethanoic acid, the precipitate was resuspended in sterilized PBS and stored at -80 ºC until used.

Tuberculin response

BCG at 2.1×10^{7} CFU/fish or FKC at 2.0×10^{8} CFU/fish were used for this vaccine study. Four weeks after vaccination, the vaccinated fish were injected with PPD intramuscularly and the kidneys were sampled at 1 and 3 days post-injection. Total RNA was extracted and cDNA was synthesized as described above and the cDNA was used for qPCR.

The changes in mRNA levels of IL-1β, IL-6 and TNFα genes after PPD injection were determined by qPCR, as described above.

Challenge test

The fish were vaccinated with BCG at 3.0×10^{8} CFU/fish (23 individuals) or FKC at 1.6×10^{7} CFU/fish (27 individuals) for 4 weeks before challenging with live *Mycobacterium* sp. strain 012971 (1.5×10^{7} CFU/fish). The number of fish used in this experiment was shown in Table 2. A colony of strain 012971 on the 1 % Ogawa medium were scraped and suspended in sterilized PBS using grass homogenizer and CFU was determined as described above. One hundred microliters of the bacterial suspension was injected into the fish intramuscularly. The fish were then maintained in 60 L tanks with circulation systems kept at 25 ºC and fed every other day. Cumulative mortality of the fishes were recorded for 12 days and relative percent survival (RPS) was calculated using the following equation; RPS = (1-[% loss of vaccinated fish/% loss of PBS control fish]) ×100.

Data analysis

Inflammatory cytokine gene expression levels during the immediate immune response and tuberculin response experiments were normalized with ribosomal protein L10 expression. In addition, the gene expression values for each group were represented as fold change relative to the value of the PBS injected group at day 1. Statistical analysis was performed with *t*-test between gene expression levels of each vaccinated groups and those of the PBS control groups at day 1.

Results

Immediate immune response after vaccination

Gene expression levels of inflammatory cytokines including IL-1β, IL-6 and TNFα in the fish injected with BCG, FKC and PBS were determined to analyze the immediate immune response at 1, 3 and 7 days post-vaccination by qPCR (Fig. 1). Gene expression levels of the inflammatory cytokines were up-regulated by BCG or FKC vaccinated fish. The IL-1β gene expression level increased by 12-fold in the BCG-vaccinated group and 8-fold in the FKC-vaccinated group at 3 days post-vaccination. The expression level of IL-6 increased 3-fold in the BCG-vaccinated group at 1 and 3 days post-vaccination. In contrast, the FKC-vaccinated group showed 3-fold increase in IL-6 gene expression level at only 3 days post-vaccination. The expression level of TNFα increased 4-fold and 3-fold in the BCG-vaccinated group at 3 and 7 days post-vaccination, respectively. In contrast, the FKC vaccinated-group showed 3-fold increase in gene expression level only at 3 days post-vaccination.

Tuberculin response

Cytokine gene expression levels were significantly up-regulated in only the BCG-vaccinated group (Fig. 2). In the BCG-vaccinated group at 1 and 3 days post-injection of PPD, IL-1β gene expression levels increased by 15-fold and 8-fold, respectively, and TNFα gene expression levels increased by 4-fold and 2-fold, respectively. In contrast, IL-6 gene expression level in BCG-vaccinated group increased by 5-fold at only 3 days post-injection of PPD.

Challenge test

Challenge experiments were performed using strain 012971 (2.4×10^{8} CFU/fish), 4 weeks after the fish were injected with BCG, FKC or PBS. Twelve days after the challenge, cumulative mortality of BCG and FKC vaccinated fish and PBS injected fish were 56.5 %, 74.1 % and 90.5 %, respectively. RPS of the BCG and FKC vaccinated groups were 37.5 and 18.1, respectively (Table 2).

Table 2

Cumulative percent mortality (%) and calculated RPS values of BCG- and FKC- vaccinated fish.

Group	No. of dead fish/total fish	Mortality (%)	RPS[a]
BCG	**13/23**	**56.5**	**37.5**
FKC	**20/27**	**74.1**	**18.1**
PBS	**19/21**	**90.5**	**—**

[a] Relative percent survival = (1-[% loss of vaccinated fish/%loss of PBS control fish]) ×100

Discussion

The Japanese flounder's immediate immune response to the two vaccine candidates against *Mycobacterium* sp. were evaluated by quantifying the inflammatory cytokine gene expression levels. In general, the inflammatory cytokine gene expression levels in the BCG-vaccinated group were higher than those in the FKC-vaccinated group during the immediate

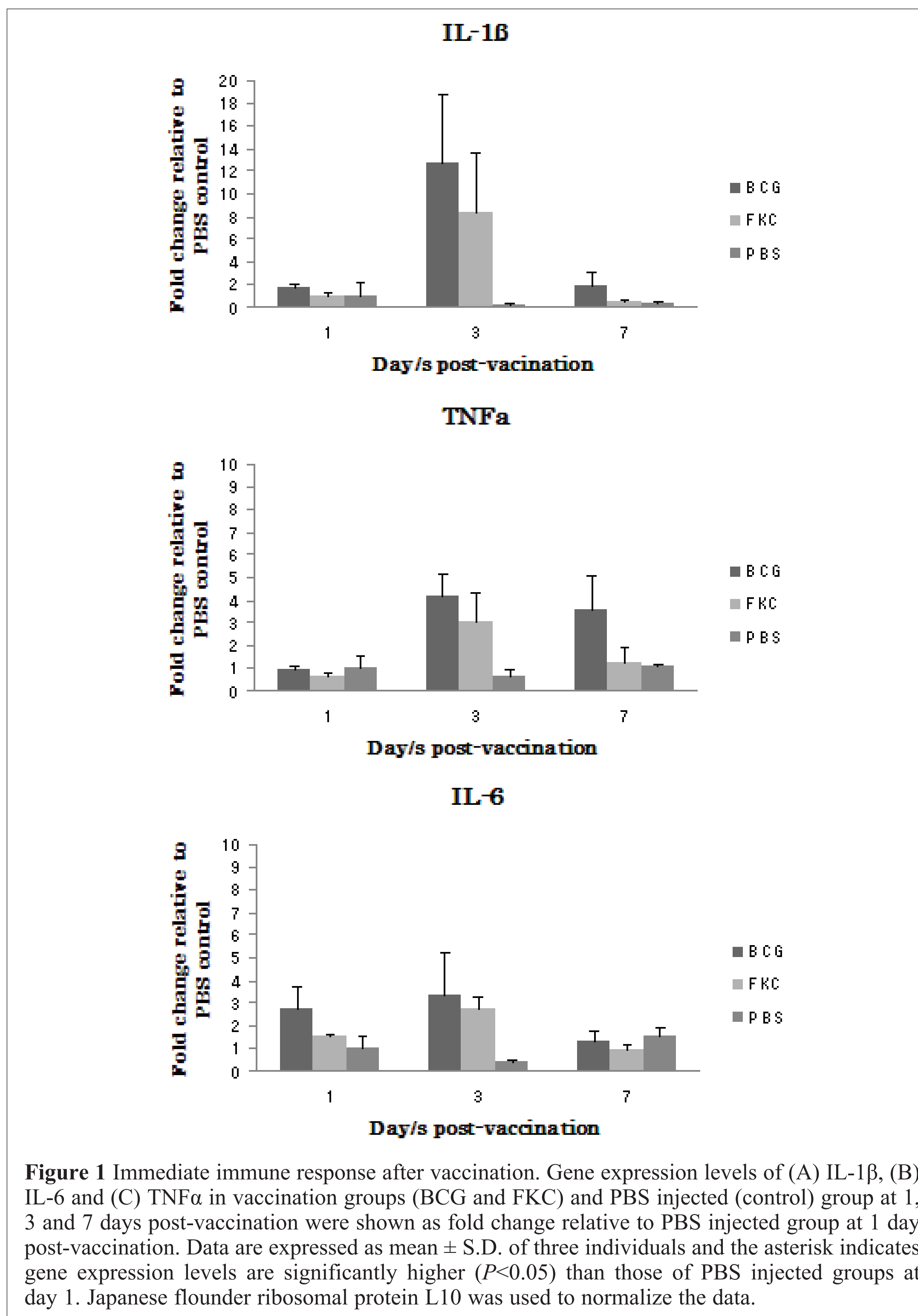

Figure 1 Immediate immune response after vaccination. Gene expression levels of (A) IL-1β, (B) IL-6 and (C) TNFα in vaccination groups (BCG and FKC) and PBS injected (control) group at 1, 3 and 7 days post-vaccination were shown as fold change relative to PBS injected group at 1 day post-vaccination. Data are expressed as mean ± S.D. of three individuals and the asterisk indicates gene expression levels are significantly higher ($P<0.05$) than those of PBS injected groups at day 1. Japanese flounder ribosomal protein L10 was used to normalize the data.

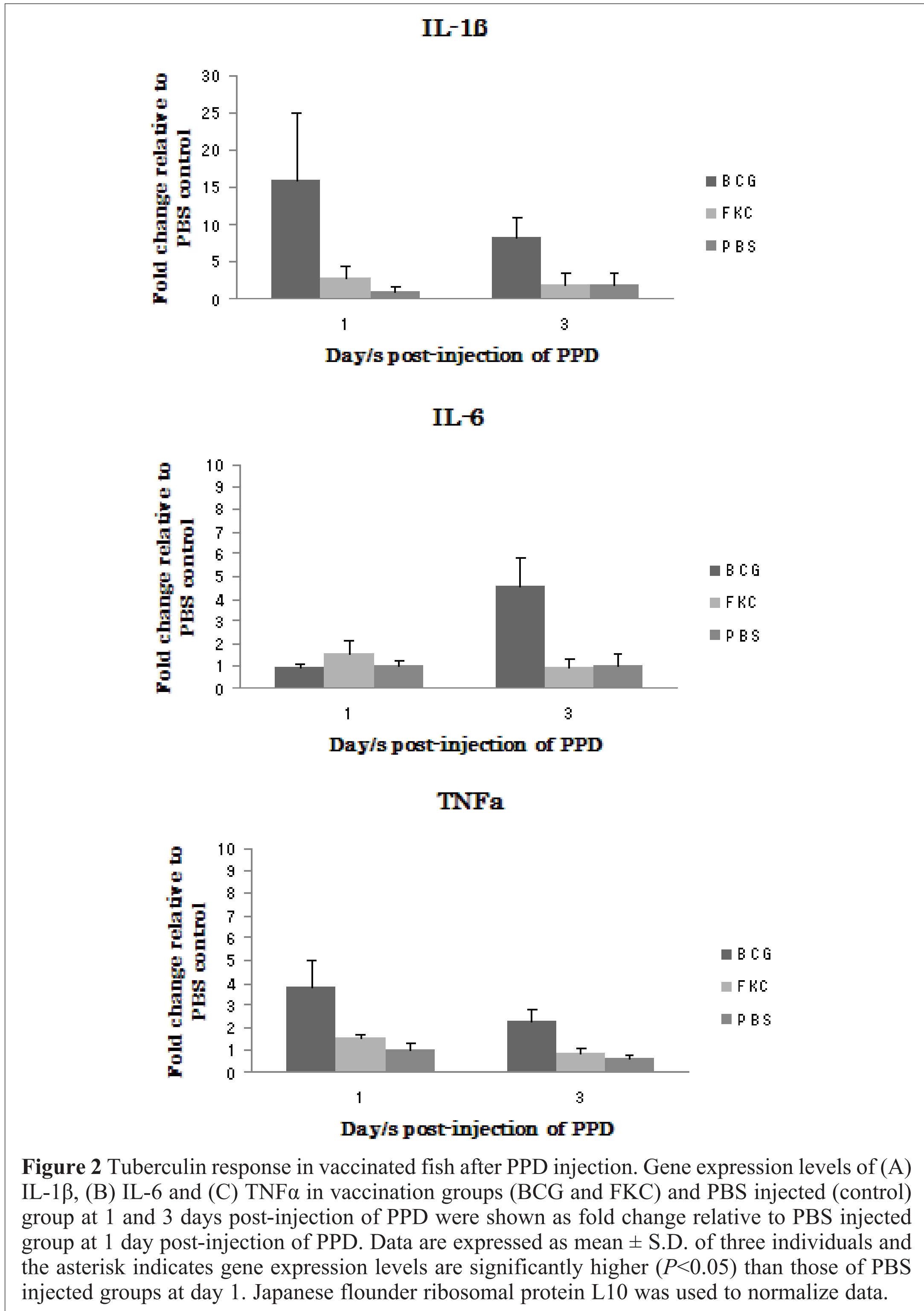

Figure 2 Tuberculin response in vaccinated fish after PPD injection. Gene expression levels of (A) IL-1β, (B) IL-6 and (C) TNFα in vaccination groups (BCG and FKC) and PBS injected (control) group at 1 and 3 days post-injection of PPD were shown as fold change relative to PBS injected group at 1 day post-injection of PPD. Data are expressed as mean ± S.D. of three individuals and the asterisk indicates gene expression levels are significantly higher ($P<0.05$) than those of PBS injected groups at day 1. Japanese flounder ribosomal protein L10 was used to normalize data.

immune response. Akira (2003) claimed that mycobacterial secreted components such as 19-kDa lipoprotein are important for the activation of the innate immune system in mammals through TLR2. These components are only produced and secreted by live bacteria such as BCG, but not by FKC. Thus, this might explain the greater up-regulation of cytokines in the BCG-vaccinated group.

In the BCG-vaccinated group, the up-regulation of TNFα was maintained until 7 days post-vaccination. TNFα plays several critical roles during mycobacterial infection. In mice infected with *M. bovis* BCG, TNF was found to inhibit the spread of the bacteria (Kindler *et al.*, 1989). It has also been reported that TNF and lymphotoxin α (LT α) signaling are required to activate cells of the immune system (Jacobs *et al.*, 2000). The loss of TNF-LTα genes in mice resulted in high susceptibility to BCG infection (Jacobs *et al.*, 2006). Therefore, the long term up-regulation of TNFα gene in Japanese flounder suggested that the host may have induced cell-mediated immune response against *M. bovis* BCG.

DTH reactions to bacterial antigens have been observed in lamprey, elasmobranches, chondrosteans and teleosts (Nakanishi *et al.*, 1999). Bartos and Sommer (1981) have also reported on DTH skin reactions against *M. tuberculosis* and *M. salmoniphilum* in rainbow trout (*Oncorhynchus mykiss*). In this study, DTH reaction was used to test whether vaccinated fish achieved specific cell-mediated immunity against *Mycobacterium* sp. antigens. Only BCG vaccinated fish showed up-regulation of the inflammatory cytokines gene expression at 1 or 3 days post-injection of PPD. During the elicitation phase of DTH, IL-1β and TNFα play major role in the efficient priming of T cells and the activation of dendritic cell (Nambu, Nakae and Iwakura, 2006), inducing chemokine production leading to an influx of other inflammatory cells (Harumi and Goldman, 2007). In contrast, IL-6 has a pivotal role in decreasing swelling at the local site to suppress or heal inflammation during the late phase of DTH (Mihara *et al.*, 1991). These results suggested that a DTH response similar to the DTH response in mammals occurred in Japanese flounder and that the BCG vaccine induced specific cell-mediated immunity against the antigen of *Mycobacterium* sp.

BCG can protect against various mycobacteriosises such as tuberculosis, leprosy, Buruli ulcer and paratuberculosis (Heinzmann *et al.*, 2008) in mammals. All mycobacteria possess mycolic acid, a component of the cell wall. CD1 molecules present mycolic acid as a lipid antigen to restricted $CD8^+$ T cells (Beckman *et al.*, 1994). Sugita (2006) suggested that the wide-spectrum efficacy of BCG is dependent on the response of CD1-restricted T cells to the lipid antigen. In this study, BCG vaccine was more effective at protecting the fish from *Mycobacterium* sp. infection than FKC vaccine. Hence, our data suggest that the Japanese flounder immune systems recognize and present mycobacterial common antigens such as mycolic acid and protect fish from *Mycobacterium* sp.

Although a return to virulence might have been concerned, live attenuated vaccine is useful for fish disease which is difficult to be eradicated by inactivated vaccine. Some mutant strains of pathogenic bacteria and some species related to pathogenic bacteria have been studied as live attenuated vaccines in fish. In fact, a mutant strain of *Edwardsiella ictaluri*

has been licensed as live attenuated vaccine against enteric septicemia of channel catfish in U.S.A. (Klesius and Shoemaker, 1999). In addition, *Arthrobacter* spp. has been also licensed as a live vaccine against bacterial kidney disease of salmonids in North America and Chile (Griffiths, Melville and Salonius, 1998). BCG vaccine has been used in mammals for a long time and shown stability of the avirulence (Calmette and Plotz, 1929), BCG vaccine may be useful against mycobacteriosis also in aquaculture industry.

In conclusion, intramuscular vaccination of Japanese flounder with BCG vaccine induced an immediate immune response and specific cell-mediated immune response against the antigens of *Mycobacterium* sp. Furthermore, BCG confers some protection efficacy against *Mycobacterium* sp. infection for Japanese flounder. It is likely that BCG will also be useful as a vaccine for other aquaculture species.

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Appendix 1

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