PRELIMINARY STUDY OF THE SOURCE OF *AEROMONAS HYDROPHILA* INFECTION ON *PANGASIUS HYPOPHTHALMUS* LARVAE

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Abstract

One of the main problems that has to be faced by *Pangasius hypophthalmus* breeders is the high mortality of the larvae, which can be a consequence of both cannibalism and bacterial diseases. The aim of this study was to explore the possible sources of *Aeromonas hydrophila* that may result in an infection of *Pangasius hypophthalmus* larvae. Isolation of bacteria were made from water source, *Artemia* culture medium, *Artemia* nauplii used as feed, ova (unfertilised eggs), sperm, and fed as well as unfed larvae that were collected every day from hatching, up to the age of 7 days. Characterisation of bacteria were done on the basis of morphological, physical and biochemical characteristics. The serological method of identification using polyclonal *Aeromonas hydrophila* antiserum was used. The results indicated an absence of bacteria from sperm. By contrast, bacteria *Alcaligenes* sp. were isolated from source of water, unfertilised eggs and from 1-day-old larvae. *Proteus* sp. were isolated from the 1st day of age in fed larvae and from the 7th day in unfed larvae. *Aeromonas hydrophila* were isolated from larvae of 2 days of age, and *Aeromonas punctata* from 2-days-old unfed larvae, meanwhile *Plesiomonas shigelloides* were identified from fed larvae of 2 and 4 days of age, and from 2-days-old unfed larvae.

INTRODUCTION

During the last few years, fish health problem became a major concern to aquaculturist in all over the world. In south-east Asian countries, fish production was badly affected by the outbreak of fish disease, such as Epizootic Ulcerative Syndrome (EUS) in 1980. Important bacterial fish pathogens, including *Aeromonas* spp., *Pseudomonas* spp. and *Flexibacter columnaris,* are regularly isolated from fish and become primary pathogenic agents frequently reducing the production of cultured freshwater fish. In late 1980, a total of 125 tons of carp were lost in Java (Indonesia) due to bacterial disease infection (Djajadiredja *et al.*, 1983). The disease was caused by bacterium *Aeromonas hydrophila.* This bacterium was not only causing mortality on common carp but also on catfishes and snakehead fish. In Indonesia, the outbreak of this disease was firstly reported from West Java but since then it has spread out to all over Java, Kalimantan and Sumatra.

Catfishes are important commonly cultured freshwater fish species besides common carp and tilapias. Intensive culture of walking catfish (*Clarias batrachus*) in Indonesia is already practised. Fish farmers are encouraged to culture catfish because it is highly profitable. African catfish (*Clarias gariepinus*) was introduced into Indonesia and is now being cultured extensively. *Clarias gariepinus* grow faster than the local species and is therefore preferred by farmers for culture. However, the development of catfish culture in recent years has been hampered by the frequent occurrence of bacterial disease.

Another species of catfish that was introduced to Indonesia 26 years ago is the Thai catfish (*Pangasius hypophthalmus*). This species of catfish resemble to local pangasiid species. Because of breeding technique of local *Pangasius* has not been developed, the Thai catfish has been cultivated as a substitute to local species. One of the main problems that has been faced by the breeder is the important mortality of the larvae, which is suspected to be a consequence of both cannibalism and bacterial disease (Subagja *et al*, 1998).

Fish diseases are often associated with intensive fish culture. Interaction leading to bacterial disease in fish depends on the presence of the pathogen, the quality of environment and the general status of the fish. Balance of these conditions can ensure fish health without the use of chemotherapeutic agent. Health is promoted by ensuring good water quality, appropriate stocking densities and providing balanced feed.

Bacterial haemorrhagic septicaemia due to strain of *Aeromonas hydrophila* may be transmitted through the water, diseased and healthy fish, other affected vertebrates, and favoured by external as well as internal parasites. A reservoir of potential pathogens probably exists in all natural and artificial water bodies (Newman, 1982).

The present paper aims at a preliminary exploration of the possible sources of *Aeromonas hydrophila* that may result in infection of *Pangasius hypophthalmus* larvae.

MATERIALS AND METHODS

Pangasius hypophthalmus used for the experiment were bred at the Research Institute for Freshwater Fisheries (RIFF) in Sukamandi, Subang West Java.

The larvae were obtained from 3-5 years old *P. hypophthalmus* brooders held in earthen ponds and supplied with water from irrigation. Fish were artificially induced to ovulated using Ovaprim and eggs were fertilised using the procedure described by Legendre *et al.* (1999). Twelve hours after hatching, the larvae were individually counted and transferred to the experimental facility. they were reared in stagnant spring water and maintained in fibreglass container and aquarium.

Isolation of bacteria

The isolation of bacteria was made from different sources:

- water (ground water which is normally used for larval rearing),
- the medium of *Artemia* culture,
- *Artemia* nauplii themselves,
- unfertilised eggs collected just after stripping of females,
- from sperm collected just after stripping of males,
- from fertilised eggs in incubation, collected at 2 h after fertilisation.

Larvae were collected every day, from hatching up to the age of 7 days. Two groups of larvae were followed, one group was totally starved during the whole duration of experiment and the other one was fed with *Artemia* nauplii. This was done in order to test for a possible direct or indirect disease transmission through feeding. Fed larvae received *Artemia* nauplii in excess, starting from 36 hours after hatching up to 8 days of age. The feeding frequency was of 8 meals per day at 09:00, 12:00, 15:00, 18:00, 21:00, 24:00, 03:00 and 06:00.

Isolations of bacteria from water, and *Artemia* medium were carried out by serial dilution-agar plating procedure. A total of 25 µl of water or *Artemia* medium was inoculated onto melted Tryptic Soy Agar (TSA) (Dipco) and then plated onto sterile Petri dish.

Isolation of bacteria from sperm was done by inoculation of an aliquot onto the TSA plate.

The surface of unfertilised eggs, fertilised eggs, larvae at different ages either fed or unfed larvae and *Artemia* nauplii, were separately sterilised by washing with ethanol 70% and then re-washed with sterile water. Each specimen was then crushed by using a sterile tissue grinder and then inoculated onto TSA plate.

All isolations were made in three replicates and incubated at 28°C.

Identification of bacteria

Identification of *Aeromonas hydrophila* was done by serological method using polyclonal *Aeromonas hydrophila* antiserum. Antiserum used was prepared from *Aeromonas hydrophila* strain No. 26 of RIFF collections, produced by a standard procedure (Burrell, 1979).

Identification of other bacteria was carried out by morphological, physical and biochemical characterisation as describes by Cappucino and Sherman (1987) and Cowan (1985). Gram stain was done to determine the shape of bacteria and gram reaction. Motility of bacteria was determined by the "hanging drop" method.

RESULTS AND DISCUSSION

A number of colonies were observed after the isolation and 12 h of incubation. The colour of the colonies varies from buff to yellowish. The results after further identification indicated that various bacteria were able to be identified. All isolated bacteria belonged to the genera *Alcaligenes, Proteus, Plesiomonas* and *Aeromonas*. Isolated and identified bacteria are listed in Table 1.

Alcaligenes sp. identified from unfertilised eggs was considered as coming from the water source, in which it was also found. So, in that case, the surface sterilising process was not properly done.

Source of sample	Isolated bacteria
Water	*Alcaligenes* sp
Sperm	None
Ova	*Alcaligenes* sp
Fertilised eggs	None
Artemia culture medium	None
Artemia nauplii	None
Unfed larvae	
Age 2	*Plesiomonas shigelloides, Aeromonas punctata*
Age 7	*Proteus* sp.
Fed larvae	
Age 1	*Proteus* sp., *Alcaligenes* sp.
Age 2	*Aeromonas hydrophila, Plesiomonas shigelloides*
Age 4	*Plesiomonas shigelloides*

Table I: Isolated and identified bacteria from water, *Artemia* culture medium, *Artemia* nauplii and *Pangasius hypophthalmus* larvae.

Bacterium *Alcaligenes* sp. was also isolated from the samples. The bacterium was isolated from water, then found in fertilised egg and finally isolated in 1-day-old larvae. According to Kersters and De Ley (1963), this bacterium occurs in water and soil. Recently Austin *et al.* (1981) described a new group (subspecies) of bacteria isolated from moribund lobster and named them as *Alcaligenes faecalis* subsp. *homari*

Some of isolated bacteria were gram positive, and cocci shape. Those bacteria were considered to be non-pathogenic bacteria. As revealed by Richard and Roberts (1978), the majority of fish pathogens are Gram-negative rod but there are some Gram-positive pathogens, including a few which are acid fast.

There was no *A. hydrophila* isolated from water, sperm, ova, fertilised eggs, nor from 1-day-old fed and unfed larvae. The absence of bacterium *A. hydrophila* in the water reservoir, sperm, ova, fertilised eggs and *Artemia*, revealed that those sources were not at the origin of infection of larvae. Also *A. hydrophila* were absent in 1-day-old larvae. However, *A. hydrophila* were discovered to infect the larvae aged of 2 days. This could be coming from other sources such as planktonic organisms, fish parasites or other organisms, as Newman (1982) stated that a reservoir of potential pathogens probably exists in all natural and artificial water bodies. Besides the role of external and internal parasites in the transmission of the disease is probably much greater than it is generally assumed. Dombrowski (1953) isolated *A. liquifaciens* from copepods (*Argulus foliaceus*) and from leeches (*Piscicola geometra*). The state of contamination of equipment and operators may also play an important role in the transmission of disease agents.

Other species of bacteria predominantly isolated from the samples was *Plesiomonas shigelloides*. According to Habs and Schubert (1962), this bacterium was formerly classified in the genus *Pseudomonas* and then transferred to the genus *Aeromonas*. This was followed by its transfer to the newly created genus *Plesiomonas*. The bacterium occurs in fish and other aquatic animal.

CONCLUSION

The transmission of disease agent seems to be not through the water source, ova, sperm, *Artemia* culture medium or *Artemia* nauplii but could be due to contamination of equipment and operators.

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PRELIMINARY RESULTS OF THE STUDY OF PARASITIC AND RED SPOT DISEASES ON HIGH ECONOMICAL VALUABLE CATFISH SPECIES IN THE MEKONG DELTA

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Abstract

The parasitic diseases and bacteria causing red spot disease in the Mekong Delta were studied in three catfishes used in aquaculture: *Pangasius bocourti*, *P. hypophthalmus* and *Clarias* hybrid (*C. macrocephalus* x *C. gariepinus*). *Ichthyophthirius multifiliis*, *Trichodina* sp., *Dactylogyrus* sp., *Gyrodactylus* sp. and *Oodinium* sp. are the parasites most often found in skin, fins, and gills of diseased fish, particularly in fry nursed in cement tanks and earthen ponds. The "white spots" disease caused by *Ichthyophthirius multifiliis* occurred in both *Pangasius bocourti* and *P. hypophthalmus*. Red spot disease has also been recorded as the most common disease on catfish at the grow-out stage in cage culture. Most species of bacteria were found to be Gram negative and motile Aeromonads such as: *Aeromonas hydrophila*, *A. caviae*, *Pseudomonas fluorescens*, *Edwadsiella tarda* and *Vibrio* sp.

INTRODUCTION

As wild fish stocks tend to decline continuously, there is an increasing need to expand aquaculture in order to satisfy the demand for high quality protein to feed the world's growing population. To obtain high productivity, the diseases of cultured species need to be understood and controlled. In this prospect, some fast growing culture fish need to be considered and studied such as *Pangasius* and *Clarias* genera that were cultured commonly in Mekong Delta. Their main favourable characteristics for aquaculture are the ability to tolerate high stocking densities, fast growth and high yield as well as their good palatability and high market value. As the farming intensity increases, diseases, which are normally present in wild populations, become much more evident in the confined condition. Fish diseases caused by pathogenic organisms such as protozoa, fungus, bacteria and virus often occur during the culture period. However, a particular attention should be given to parasitic and bacterial diseases, as they have been considered as a serious problem on catfish.

In some cases, outbreaks of bacterial diseases have been related to stress factors. Circumstantial evidence suggests that *Aeromonas hydrophila* may be a secondary invader of parasite induced injuries (Kumar, 1986; cited by Pai *et al.*, 1995). In addition, many bacterial and parasitic pathogens have been reported in the Walking catfishes *Clarias batrachus*, *C. macrocephalus*, and the hybrid *C. macrocephalus* x *C. gariepinus* (Tonguthai *et al.*, 1993; Angka, *et al.*, 1994). The symptoms of the disease are similar in appearance to those found in other bacterial haemorrhagic septicaemia and differentiated into three main categories: acute with few gross symptoms, acute form with dropsy, chronic ulcerous form. It is believed that *A. hydrophila* is an opportunistic organism that contributes to secondary infection of the lesions; this bacterium has been also isolated from Epizootic Ulcerative Syndrome (EUS) (Anonymous, 1986; Llobrera & Gacutan, 1987). However, Supriyadi *et al.* (1995) indicated that Walking catfish (*C. batrachus*) can be protected from *A. hydrophila* by vaccination.

Much of the previous effort in studying and controlling diseases on catfishes such as *Pangasius bocourti*, *P. hypophthalmus* and the hybrid *Clarias macrocephalus* x *C. gariepinus* in Vietnam have been dissipated through a lack of complete scientific data. Therefore, the study of parasitic diseases and bacteria causing red spot disease (bacterial haemorrhagic septicaemia) on these catfish of economical importance have been considered as an important goal in the project. This paper presents the preliminary results obtained in this field.

MATERIALS AND METHODS

Studied sites: the survey was conducted in Chau Doc town (An Giang province), Vinh Long and Can Tho provinces. Samples of *Pangasius* at the grow-out stage were collected from cage culture in Chau Doc town with three different period during both the dry and the rainy seasons. At the same time, fish farmers were interviewed using adapted questionnaire forms. Information and data related to disease, culture techniques and health management, were recorded on the farms and at the fisheries processing plant (AGIFISH CO.).

Two hatcheries, located at Can Tho University and Chau Doc town (My Chau hatchery), were involved in the propagation and nursing of *P. bocourti* and *P. hypophthalmus* in the Mekong Delta. Fry and fingerling samples with parasitic diseases were collected from indoor cement tanks and examined every month.

Study of pathogens: method of study of fish parasites of Dogiel (1933) was applied in this study. The following methods were used for the collection of fish samples: (1) live diseased fish were kept in nylon bags and stored in ice container, samples being analysed as soon as possible (within 24 hours); (2) fish samples were transported to the laboratory in nylon bags supplied with oxygen; (3) diseased fish were also processed on the field, Tryptone Soy Agar plates (TSA) was then used for initial inoculation. Rimler-shortts Agar (RS), Decarboxylase (Arginie Lysine and Ornithine), Oxidation fermentation medium (O/F), Triple Sugar Iron Agar were used for biochemical tests to identify bacteria. The work was carried out at the Fish disease laboratory of the College of Agriculture, Can Tho University. Fish bacteria were studied according to the methods of Plumb (1983) and Frerich (1984).

RESULT AND DISCUSSIONS

Parasitological study: the commonest ecto-parasites were recorded on the cultured catfish studied (Table 1). Some protozoan (*Trichodina* sp, *Oodinium* sp), were observed on *Clarias* hybrid fry in earthen ponds and on *P. bocourti* fingerling in nursing cement tanks. White spot disease caused by *Ichthyophthirius multifiliis* occurred in both *P. hypophthalmus* and *P. bocourti*. In addition, Monogenetic trematodes consisting of *Dactylogyrus* and *Gyrodactylus* sp, attacked skin and gills causing high mortality on small fish, during the rainy season. Flashing movements, pin-point haemorrhages, excessive mucus production, obstacle to oxygen uptake, impaired feeding and lethargy have been commonest clinical signs observed. Specific signs as "white spots" were caused by *Ichthyophthirius multifiliis*.

The mortality rate caused by parasitic diseases

Infected catfish	Location	Stage	Parasites	No. of fishes tested	Degree of infestation
P. bocourti	Gills, fins and skin	Fingerlings	*Ichthyophthyrius*	40	++++
	Gills, fins and skin		*Trichodina*	30	++
	Skin and gills		*Dactylogyrus*	15	+++
	Gills and skin		*Gyrodactylus*	10	+++
	Gills and skin	Grow-out stage	*Dactylogyrus*	25	++
	Skin and gills		*Gyrodactylus*	10	++
	Tract intestine		*Balantidium*	7	++
	Tract intestine		*Trematoda*	11	+
	Tract intestine		*Nematoda*	6	+
P. hypophthalmus	Gills, fins and skin	Fingerlings	*Trichodina*	20	+++
			Ichthyophthyrius	14	++++
			Dactylogyrus	5	++
			Gyrodactylus	5	++
Clarias hybrid	Skin, fins, gills and muscle	Fingerlings	*Trichodina*	50	+++
			Ichthyophthyrius	20	+++
			Oodinium	10	++
		Juveniles	*Metacercaria*	5	+

\+ = light, ++ = medium, +++ = heavy, ++++ = very heavy

Table 1: Parasites found on cultured catfish in the Mekong delta.

reached 80-90% at hatchery of Can Tho University and 50% at My Chau hatchery in Chau Doc town. *Ichthyophthirius multifiliis* was responsible for most of the mortality observed in *P. hypophthalmus* and *P. bocourti*. In general, external parasites attack skin, fins, and the gills causing acute mortality due to their direct life cycle or rapid multiplication. Unbalanced diets also appeared as one of the reasons for diseases and mortality of catfish.

In addition, *Balantidium* and Nematodes were observed in the intestinal tracts of *P. hypophthalmus* and *P. bocourti* and *Clarias* hybrid. These parasites did not cause high mortality on catfish. Immature Digenea (metacercaria) were also found under skin or muscle.

Bacteria isolated from diseased fish: seven strains of *Aeromonas hydrophyla*, three strain of *A. caviae* and *Pseudomonas sp.* were identified. These bacteria caused red spot disease on *P. bocourti, P. hypophthalmus* and *Clarias* hybrid. All strains of bacteria isolated from these catfishes are given in Table 2.

Besides pathogens, several other factors were responsible for outbreaks of disease in catfish farms. They include particularly overcrowding, poor environment quality, unbalanced diets and poor sanitary measures. Though the development of aquaculture and the desire to increase farm production, farmers often forget the importance of maintaining the delicate balance between the host and the environment. In hatcheries, changes in physico-chemical and microbiological quality of water also generate stress in fish making them more susceptible to invasion by pathogens. Subasinghe (1995) shown that the best quality environment with respect to an aquatic organism refers to water body close to its natural environment. This includes provision of good physical, chemical and microbial quality of water, adequate swimming space and feeding with a nutritionally balanced diet.

CONCLUSIONS

White spot disease (Ich) is still a serious problem in small catfish farming, including fingerling and even juvenile stages. Therefore, study of chemotherapeutants application methods, as well as environment management for prevention and treatment should be carried out. Prevention and control of fish disease through environmental manipulation is far more economical and effective than other methods of control. Therefore, attention should be paid for water treatment in nursing tank systems in hatcheries, in order to reduce mortality caused by ectoparasites. Unbalanced diets may be related to outbreak of some diseases, especially lack of vitamin and specific minerals in diets. An experiment on supplying Vitamin C for prevention of white spot disease should be carried out in near future. Hemorrhage disease (red spot disease) have caused a reduced production of catfish at the grow-out stage. Study on cause, treatment and precautionary measures have to be considered.

Bacteria isolated	Site of isolation	Infected species	Clinical signs	No. of fishes examined
A. hydrophila ***A. caviae***		*P. bocourti*, *P. hypophthalmus*, *Clarias* hybrid	Haemorrhages on the muscle and internal organs or at the base of fins; red or yellow fluid in abdominal cavity and fin rot	10
Pseudomonas fluorescens	Kidney Liver Spleen Lesion	*P.bocourti*, *Clarias* hybrid		2
Vibrio sp.				2
Streptococcus.sp		*P. bocourti*	White nodules in the internal organs and protruded anus	3
Edwadsiella tarda				4

Table 2: Bacteria isolated from diseased catfishes cultured in the Mekong delta.

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