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Pathogenicity of Motile *Aeromonas* Species Isolated from Fishes with Epizootic Ulcerative Syndrome (EUS) in Southeast Asian Countries

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Abstract

The pathogenicity of *Aeromonas* species isolated from fishes with EUS was investigated in goldfish by intramuscular injection and immersion method at 20°C and 25°C. For intramuscular injection median lethal doses (LD₅₀) were varied from $2 \times 10^{6.8}$ to 6×10^6 CFU/fish at 20°C and $2.6 \times 10^{4.5}$ to 1.2×10^8 CFU/fish at 25°C. Swelling at the site of injection was observed in almost all the experimental fish from the first day which gradually damaged and from where simultaneously scales protrusion appeared following dermal lesion from 2 to 3 days after injection. Internal haemorrhagic lesions in the abdominal cavity at the chronic stage were common instances. Infections were found significantly on the mechanically injured fish by bath challenge. Dermal lesions and haemorrhagic lesions in the abdominal cavity were also found at the chronic stages, which were identical to those observed for intramuscular injection. Infection rates at 25°C in most cases were higher than those at 20°C. The challenged strains were significantly re-isolated from kidney or lesions of dead, moribund, infected or sacrificed fish from both methods. The results show that the motile *Aeromonas* spp. are capable of causing EUS in goldfish.

Key words: Pathogenicity, *Aeromonas*, EUS, Southeast Asia, Goldfish

Introduction

Motile *Aeromonas* spp. are recognized as pathogenic to poikilothermal animals, such as frogs, snakes or fish and also to homeothermal animals and humans (Russell, 1898; Altwegg and Geiss, 1989; Janda, 1991; Popoff, 1984). These species are considered to be opportunistic pathogens. In intensive or semi-intensive fish farming systems fish are stressed by mishandling, overcrowding, lack of nutrition or poor water quality. These conditions easily enhanced diseases in aquatic animals especially those caused by *Aeromonas* species. Although the motile aeromonads are causing diseases in fish, only the non-motile *A. salmonicida*, is generally regarded to cause furunculosis and septicemia (McCarthy, 1977) worldwide. There are increasing evidence that the motile aeromonads are causative agents of fish diseases. *Aeromonas hydrophila* and *A. sobria* have frequently been isolated from EUS lesions in fish²⁾ (Llobrera and Gacutan, 1987; Torres et al., 1990; Subasinghe et al., 1990; Lio-Po et al., 1990) in Southeast Asian countries. This disease causes severe mortalities of both cultured and wild fish species every year in a cyclic manner

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²⁾ Report of the expert consultation on ulcerative fish disease in Asia-Pacific region. Food and Agricultural Organization of the United Nations. TCP/RAS/4508, Bangkok (1986).

(Roberts et al., 1986; Roberts et al., 1993). It has been suggested that motile *Aeromonas* contribute to the pathogenesis of the disease (Costa and Wejeyaratne, 1989). Three motile *Aeromonas* species and one new species of the genus *Aeromonas* have been identified by phenotypic and genotypic method from EUS affected fishes described in the previous paper (Iqbal et al., 1998a). The present study was therefore, undertaken to understand the pathogenicity of the identified strains in experimentally induced goldfish (*Carassius auratus*).

Materials and Methods

Bacterial strains

Two strains from each of the three genospecies and unidentified T8 strain were selected for this study (Table 1).

Table 1. Details of EUS strains used in this study

Strain No.	Genospecies*	Host fish species	Origin country	Organ	Year
M29	<i>A. hydrophila</i>	<i>Oreochromis</i> sp.	Malaysia	Kidney	1987
T20	<i>A. hydrophila</i>	<i>Puntius gonionotus</i>	Thiland	Liver	1995
B1	<i>A. veronii</i> biotype <i>sobria</i>	<i>Cirrhinus mrigala</i>	Bangladesh	Lesion	1994
M16	<i>A. veronii</i> biotype <i>sobria</i>	<i>Cyprinus</i> sp.	Malaysia	Lesion	1987
B2	<i>A. jandaei</i>	<i>Ci. Mrigala</i>	Bangladesh	Lesion	1994
M34	<i>A. jandaei</i>	<i>Aristichthys nobilis</i>	Malaysia	Kidney	1987
T8	<i>Aeromonas</i> sp.	<i>Osphronemus groamy</i>	Thailand	Kidney	1994

* See reference Iqbal et al. (1998a).

Experimental fish

Goldfish (*C. auratus*) weighing 12–20 g, purchased from a local pet shop, were used in this study. Prior to the experiment, fish were acclimatized to laboratory conditions at either 20°C or 25°C for at least two days.

Preparation of bacterial suspension

The strains were cultured on a nutrient agar (NA; polypepton 10 g, meat extract 5 g, NaCl 1.2 g, agar 15 g, distilled water 1,000 ml, pH 7.2) at 25°C for 24 h prior to the experiment. They were then suspended in sterile physiological saline (0.85% NaCl) and desired dilutions were prepared by ten-fold dilution method.

Pathogenicity test

The fish was lightly anaesthetized in MS222 (ethyl m-aminobenzoate methanesulfonate) prior to challenge. Then the fish were injected intramuscularly with 0.05 ml of three different concentrations of bacterial suspension in the flank, just below the anterior part of the dorsal fin. Negative control fish received 0.85% physiological saline instead of bacterial suspension. Each suspension of strain was injected to five fish. Each group was kept separately in a 3 l aquarium with aeration at 20°C and 25°C for 7 d and the water of each aquarium was changed at 24 h interval. No feed was given during the experiment. The fish were monitored

daily and mortality noted. The median lethal dose (LD₅₀) was calculated according to the method of Reed and Muench (1938).

The most pathogenic strain of each genospecies and the *Aeromonas* sp. (T8) obtained by intramuscular injection were selected for the bath challenge. Five mechanically injured fish (some scales were removed from the anterior part below the dorsal fin and one scratched with a sterile explorer) were immersed approximately in the concentration of median lethal dose of the 4 *Aeromonas* species separately for 20 minutes. Then the challenged fish were washed with distilled water and maintained under the same condition as described above. Mortality and infection rate were monitored daily for two weeks. The surviving fishes were sacrificed after the course of experiments in both challenges and the respective strains were re-isolated from the lesions or kidney by swabbing.

Re-isolation of challenged strains

The re-isolates from kidney and lesions of dead, moribund or sacrificed fish were purified on nutrient agar. Then colony hybridization was performed according to the method of Mass (1983) with minor modifications to confirm the re-isolates. Briefly, DNAs from seven strains used in this study were extracted and labeled with photobiotin as described previously (Iqbal et al., 1998a). The re-isolates were spot cultured on nutrient agar plate. After overnight grown at 25°C, a sterilized Whatman 541 filter paper was placed over the colonies for 1 h. The paper was carefully lifted out with forceps and placed colony side up on a suitable Whatman 3 MM paper soaked with 0.5 M NaOH/1.5 M NaCl in a petridish and lysed the cells at 100°C for 3 min in water bath. Then the DNAs were neutralized with 0.5 M Tris-HCl (pH 7.4)/1.5 M NaCl and washed with 2×SSC and finally dried at 37°C for about 1 h. The DNAs were then hybridized with 9.8 ml of hybridization mixture containing 40 µl of 50 µg/ml photoprobe labeled DNAs in a hybridization bag at T_m -25°C (optimal condition) and T_m -15°C (stringent condition) overnight. Hybridized DNAs were visualized by one step colorimetric detection method according to Keller and Manak (1989).

Results

Swelling at the site of injection was observed in all the experimental fish from the first day which gradually damaged and where simultaneously scales protrusion appeared following dermal lesion from 2 to 3 days after injection. Internal haemorrhagic lesions in the abdominal cavity at the chronic stage were common. The lesions and signs caused by challenged *Aeromonas* spp. in the experimentally infected fish were almost similar to EUS found in nature (Fig. 1). Median lethal doses (LD₅₀) were varied from $2 \times 10^{6.8}$ to 6×10^8 CFU/fish at 20°C and $2.6 \times 10^{4.5}$ to 1.2×10^8 CFU/fish at 25°C for intramuscular injection (Table 2). LD₅₀ values for the tested fish kept at 25°C were approximately one order lower than those for fish were kept at 20°C. The different challenged *Aeromonas* spp. were re-isolated from the kidney and external lesions of dead, moribund and sacrificed fish (Fig. 2). No mortality and no infections were observed in the control group.

Table 3 shows the results of the bath challenge of the different challenged strains. A significant number of fish were infected by the challenged strains.



Fig. 1. External lesion (arrow) on the trunk region by intramuscular injection with strain M16 (*A. veronii* biotype *sobria*).

Table 2. Virulence of *Aeromonas* species after intramuscular injection

Strain No.	Genospecies	Median lethal dose (CFU/fish) at	
		20°C	25°C
M29	<i>A. hydrophila</i>	$6 \times 10^{7.5}$	$9 \times 10^{6.3}$
T20	<i>A. hydrophila</i>	$6 \times 10^{7.3}$	$1.7 \times 10^{6.1}$
B1	<i>A. veronii</i> biotype <i>sobria</i>	$5 \times 10^{7.3}$	$1.8 \times 10^{6.4}$
M16	<i>A. veronii</i> biotype <i>sobria</i>	$2 \times 10^{6.8}$	$2.6 \times 10^{4.5}$
B2	<i>A. jandaei</i>	$2.3 \times 10^{7.5}$	$9 \times 10^{6.5}$
M34	<i>A. jandaei</i>	6×10^8	1.2×10^8
T8	<i>Aeromonas</i> sp.	$1.8 \times 10^{6.9}$	1.9×10^6
Control*			

* No mortality or any sign of infection were observed in the control group.

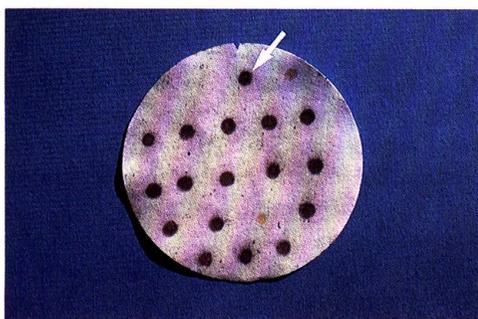


Fig. 2. A photograph of a Whatman 541 filter with colonies of re-isolates after colony hybridization with photobiotinylated M16 probe DNA. Arrow indicates the positive control; deep color (similar to positive control) indicates the positive results and fade color indicates negative results.

Table 3. Infection rates, mortality and re-isolation rates of goldfish challenged with *Aeromonas* species by immersion method

Strain No.	Genospecies	Infection rate (%) at		Mortality (%) at		Re-isolation rate (%) at	
		20°C	25°C	20°C	25°C	20°C	25°C
T20	<i>A. hydrophila</i>	80	100	0	20	100	100
M16	<i>A. veronii</i> biotype <i>sobria</i>	100	100	0	40	100	100
B2	<i>A. jandaei</i>	80	100	0	20	80	100
T8	<i>Aeromonas</i> sp.	60	80	0	0	80	80
Control		0	0	0	0	0	0

Dermal lesions and haemorrhagic lesions on the abdominal cavity found at the chronic stages which were almost similar to that observed for intramuscular injection (data not shown). Infection rates at 25°C in most cases were higher than those at 20°C. Mortality did not occur at 20°C. On the other hand, only a few fish died at 25°C when infected with *A. hydrophila* (T20) or *A. veronii* biotype *sobria* (M16) or *A. jandaei* (B2). Even though mortality was low in immersion method the re-isolates were identified from kidney or lesions of moribund or infected fish were significantly identified (Table 3).

Discussion

It is evident from the results that all strains of the three genospecies of *Aeromonas* have the potentials to cause EUS-like lesions in goldfish kept at 20 to 25°C (Table 2 and 3). The median lethal doses of the different strains used were variable. The doses varied from $2 \times 10^{6.8}$ to 6×10^8 CFU/fish at 20°C and from $2.6 \times 10^{4.5}$ to 1.2×10^8 CFU/fish at 25°C. *A. veronii* biotype *sobria* was found to be more pathogenic followed by the unidentified *Aeromonas* strain (T8 strain), *A. hydrophila* and *A. jandaei* (Table 2 and 3). Overall, the median lethal doses of the challenged *Aeromonas* spp. were about one log order lower for the fish kept at 25°C.

Even though there was no significant mortality by bath challenge but high infection rates both at 20°C and 25°C were recorded (Table 3). In all cases the same strains were re-isolated either from lesions and kidney of dead and moribund as well as sacrificed fish. The presence of the different challenged *Aeromonas* spp. in the lesions and kidney of dead and moribund and sacrificed fish indicated that they have the potentials to cause EUS in goldfish. It is suspected that these bacteria may penetrate into the kidney of the infected test fish through artificial injury, have the ability to multiply and cause disease or mortality.

The fish culture systems in Southeast Asian countries are now semi-intensive to intensive, where stocking densities are high. Besides, in the open water system, water pollution from environment is very common in the Southeast Asian countries (Iqbal et al., 1996 and 1998b). The fish may succumb to motile *Aeromonas*, the most common bacterial flora in the aquatic environments when they exposed to such stressful conditions that break down their normal immune system. Motile *Aeromonas* possesses various extracellular enzymes and pathogenic factors. These factors may produce ulcerative lesions on the body surface of the fish first and then

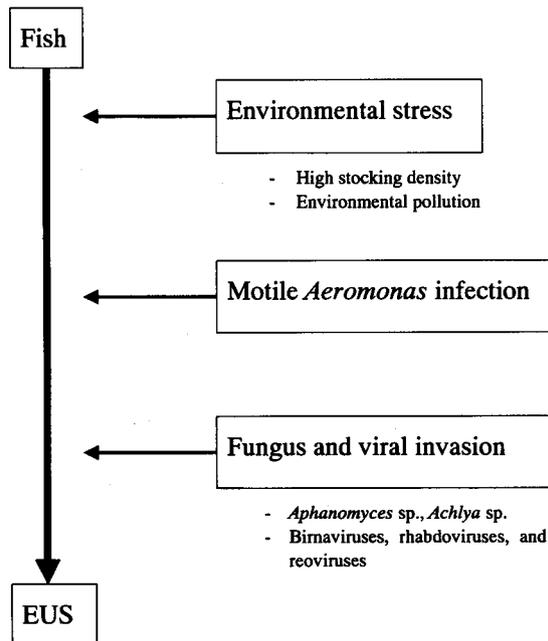


Fig. 3. A postulated story of EUS in freshwater fishes in Southeast Asian countries.

Aphanomyces and *Achlya* fungi or birnaviruses, rhabdoviruses and reoviruses invade into the lesions, which severely damage the tissues in the infected areas and cause mortality of the fish (Fig. 3).

From the results of experimental infection at 20°C and 25°C, it was confirmed that the pathogenicity of the strains to goldfish was relatively strong at 25°C (Table 2 and 3). According to Chinabut et al. (1995) an average water temperature of Southeast Asian countries is 26°C when the EUS outbreak occurs. The results of the present experiment, are therefore, corresponded to that of the temperature. This temperature may enhance the bacteria to increase themselves inside the hosts, influence to release various enzymes, which in fact causes infection or death of the fish. So, the temperature may be one of the factors of high virulence of *Aeromonas* species. However, it needs further studies to clarify the temperature to produce different enzymes and virulent factors from the strains used in this study and their roles to induce infections on fish.

The pathogenicity of the challenged *Aeromonas* spp. was not so strong in goldfish. But the challenged strains were consistently re-isolated from either lesions or kidney of the tested fish. Further studies are therefore, needed to know the pathogenicity and the pathogenic factors of the strains to EUS susceptible fish.

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