VIRULENCE OF *PSEUDOMONAS* AND *AEROMONAS* BACTERIA ISOLATED FROM *ANABAS SP* FROM LAL DIGHI,PASCHIM MEDINIPUR,WEST BENGAL

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ABSTRACT

The significance of *Aeromonas* and *Pseudomonas* bacteria in association with out breaks of diseases in feral and aquaculture fish production is of paramount important.Seven isolates of *Aeromonas hydrophila*(4) *A.voronii*(2) and *Pseudomonas aeroginosa*(1) isolated from normal and ulcer affected *Anabas sp* in Lal Dighi were examined for virulence. Invitro experiment was conducted in 10 disinfected 30L glass aquaria filled with chlorine free water.300 healthy *Anabas sp* (60-100gm) were used in which 30 fishes were stocked in each aquarium.Two aquaria stocked with 20 fishes each were used control.The fishes were acclimatized for 19 days prior to the infection experiment.Each fish except the control intramuscularly injected with 0.1 ml of the experimental bacteria (concentration, 2.3×10^8 CFU/ml) using 21/guage sterile needle.The infected fishes were observed for 19 days.The injected bacteria were then isolated from the experimental fishes and subjected to morphological, biochemical and antibiotic susceptibility tests.Result showed that ;120 out of 190 infected fishes developed clinical abnormalities such as skin darkness,scales detachment,blindness and large irregular haemorrhage on the body surface,fin necrosis,exophthalmia and eye cataract/trachoma within four days and mortality rate of 97%.The isolated strains were motile,gram(-ve) and were resistant to Ampicillin,Streptomycin,Amoxyllin and novobiocin.This study concluded that *Aeromonas* and *Pseudomonas species* are responsible for the out break of ulcerative diseases in Lal Dighi.

Key words: Pseudomonads,Aeromonads,Virulence,*Anabas sp*,Lal Dighi

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INTRODUCTION

Among the etiological agents of bacterial fish diseases *Pseudomonas* and *Aeromonas* are considered one of the most important fish pathogens.These microorganisms are responsible for ulcer type diseases including ulcerative syndrome,bacteria haemorrhagic septicemia,tail and fin rot,bacterial gill rot and dropsy[1].These bacteria have been reported to cause septicemia in *Anabas sp* in Midnapur that was more prevalent during winter period[2]

Virulent bacteria excrete tissue degrading enzymes and toxins to escape the immune defence of the host.Cell surface structure functioning as adhesion factors or having some other roles in the infection process as well as extracellular products have been studied widely in bacterial fish pathogens[1].For instance,capsular material or lipopolysaccharides are releated to virulence in *Vibrio* and *Aeromonas hydrophila*[3,4].Virulence of different Aeromonads and Pseudomonads bacterial isolates have been studied elsewhere in diseased fishes in cultured and capture fisheries[5].In addition,a review of the pathogenic gram(-ve) bacterial infections in warm water fishes was reported by[6]. In Midnapur, pathogenic experiment reported *Aeromonas hydrophila* to cause up to 100% mortality in experimental fishes (*Anabas sp*) within 42 h.Pathogenic attributes are present in a high percentage of water borne strains but their virulence for fish is lower than that displayed by strains isolated from fish[1].Studies on virulence of bacterial pathogens of fish is essential for development of new immunoprophylactic and chemotherapeutic reagents to fight bacterial infection,since the development of antibiotic resistence by bacteria has led to diseases becoming one of the major problems in the fisheries sector.Limited studies are present in *Anabas sp* fish diseases in general.To avoid losses that might arise due to emergent fish infections;investigation of pathogenic bacteria in fishes and their virulence encounter a routine significance.In this study,investigation on the virulence of *Aeromonas* and *Pseudomonas* bacteria isolated from *Anabas sp* was the main objective.

MATERIALS AND METHOD

Collection of fish:

Seven isolates of *Aeromonas hydrophila* (4),*A.veronii* (2),and *Pseudomonas aeruginosa*(1) from normal and diseased feral and cultured fishes were tested for virulence to *Anabas sp* by following the method[9].Prior to injection;healthy *Anabas sp* weighing 10-100 gm

were obtained from Lal Dighi in Midnapur,transferred and maintained in 10 glass aquaria supplied with de-chlorinated tap water with aeration and allowed to acclimatize for 19 days.

Isolated the bacteria:

To each experimental fish,0.1 ml of 2.3×10^8 CFU/ml of the isolated bacterial suspension was intramuscularly injected using 21 gauge sterile needle.Ten fishes were injected with Phosphate Buffered Saline(PBS;pH 7.2) [control(1)] using the same procedure.Another 15 fishes were held untreated[contrl(2)].The observation time was 19 days.The virulence of the strains was categorized on the basis of development of clinical signs and percentage mortalities.90%-100% mortality within 24h as highly virulent;over 50% mortality and lesions within 24-42h as moderately virulent and over 50% mortality with hemorrhagic lesions after 42h but within a specified time period of 120h as a virulent.The bacteria strains were re-isolated from the dead and from fish with clinical conditions and examined.

Anti-bacterial activity of different drugs against *Aeromonas* and *Pseudomonas* species isolated from fish:

Thirteen antimicrobial drugs were evaluated for effectiveness against *Aeromonas* and *Pseudomonas* strains using disc diffusion technique[10]. Stock cultures of the virulent strains were grown in nutrient broth for 24h at 28℃.After centrifugation at 5000g for 30min at 4℃,bacteria were re-suspended in sterile Phosphate Buffered Saline (PBS) and diluted to a turbidity equivalent to a McFarland No. 0.5 standard solution (0.5 ml $BaSO_4$ + 99.5ml 0.36N HCl).

0.1 ml of bacterial suspension was spread onto Mueller-Hinton agar (Difco) and chemotherapeutic agent discs were then added and preparation incubated at 28℃ for 24h (Bauer et al.1966).The chemotherapeutic agent used included three cell wall synthesis inhibitors (Ampicillin,15µg;Amoxicillin,30 µg; and PenicillinG,15IU); seven protein synthesis inhibitors (Chloramphenicol,40 µg; Erythromycin,20 µg; Gentamicin,15µg; Kanamycin, 40 µg; Neomycin, 40 µg; Streptomycin, 15 µg; Tetracycline, 40 µg;) and three nucleic acid synthesis inhibitors (Ciprofloxacin, 10 µg; Novobiocin, 10 µg; and Trimethoprim-sulfamethoxazole, 30 µg;) Table

2. Characterization of strains as resistant ,intermediate,or sensitive was based on the size of the inhibition zones around each disc according to standards by [11].

RESULTS

Virulence for fish

Seven bacterial strains used during the infection trial;strains 1,2.3,4 and 6 were highly virulent to the experimental fish in which they caused mortality ranging between 72-97% within 42 h (Table 1) with the dose of bacterial at LD_{50} value of 2.3×10^{8} CFU ml^{-1} , while strains 5 and 7 were classified as avirulent according to the degree of virulence described by [12]

Table 1.Virulence results for *Anabas sp* of *Aeromonas* and *Pseudomonas species* after intramuscular infections

Isolate No.	Number of fish/isolates	Fish mortality / isolate / hrs	% Virulence
1 (260S) *Aeromonas veronii*	25	21/24	97
2 (130K) *Psudomons aeruginosa*	25	20/25	92
3 (8.6Intest) *A. hydrophyla*	25	15/48	75
4 (85G) *A. veronii*	25	19/45	85
6 (85T) *A.hydrophyla*	25	17/48	85
5 (130K) *A.hydrophyla*	25	15/95	72
7 (650K) *A.species*	25	13/120	65
Control I	15	0/15	0
Control II	15	0/15	0

Clinical Observation

The results for the virulence test are summarized in Table (1).Out of 300 experimental fishes treated , 120fishes (40%) showed clinical abnormalities including skin colour darkness,detachment of the scales,large irregular hemorrhages on the body surface, shallow to deep necrotizing ulcers on the skin ,fin necrosis ,inflamed vent,exophthalmia,blindness and eye cataract/trachoma,(Plates-A-F).No of clinical abnormalities or death confirmed in the control fish.

Antibiotic Disc	Response of bacterial strains to different antibiotics				
	Strain-1 (260 Skin)	Strain-2 (130 Kidney)	Strain-3 (8.6 Intestine)	Strain-4 (85 Gill)	Strain-6 (85 Tissue)
Penicillin G	R	S	S	R	R
Kanamycin	S	S	S	S	S
Gentamicin	S	R	S	S	S
Chloramphenicol	S	S	S	S	S
Ampicillin	R	R	S	R	R
Streptomycin	R	R	R	R	R
Amikacin	S	S	I	S	S
Amoxicillin	R	I	S	R	R
Trimethoprimsufamethozole	R	R	S	S	R
Erythromycin	R	S	I	R	R
Tetracycline	R	S	R	S	S
Ciprofloxacin	S	R	S	S	S
Novobiocin	S	S	R	S	S
Neomycin	R	S	S	S	S
Summary S/R	6/8	8/6	9/5	9/5	8/6

Key word: R- Resistant; S- Susceptible; I- Intermediate based on the size of the inhibition zone around the disc as described in [11]

Anti-microbial sensitivity test:

The results of antimicrobial tests revealed that most of the tested drugs (57.66%)were effected against the re-isolated strains. This provides a wide range of drugs for fish for fish farmers to obtain treatment to their fish in case of bacterial disease outbreaks that is associated with *Aeromonas* or *Pseudomonas*. About 42.34% of the drugs were not effective to the re-isolated strains.

DISCUSSION

Although the experiment involved seven isolates,only five were found to be virulent; the other two isolates (*A.species* and *A.hydrophila* from the kidney) were either avirulent or weakly virulent according to the degree of virulence described by [12].Using morphological, physiological and biochemical characteristics;strain 4 was identified as *P.aeruginosa*. *P .aeruginosa* is reported to be the only gram(-)ve bacillus capable of producing distinctive water soluble pigment, pyocyanin [13];strains 1 and 2 were confirmed as *Aeromonas veronii* whereas strains 3 and 6 were *Aeromonas hydrophila* [14].Both strains were considered to be virulent as they caused clinical abnormalities with mortality above 72% within 42h.The isolated bacterial species are reported to cause haemorrhagic septicaemia and ulcerative diseases in finfish in Lal Dighi and selfish elsewhere [1,5,14-17].

From the bacterial challenge experiments, a dose of bacteria at LD50 value of 2.3×10^8 CFU ml^{-1} of strains *A.veronii* (2), *Pseudomonas aeruginosa* (1), *A. hydrophila subsp dhakensis* (1) and A. hydrophila (3) were able to cause 75% to 97% mortalities within three days while strains of *A. hdrophyla* and *Aeromonas species* from the Kidney caused up to 72% mortalities after three days but within the specified period of four days. Although the physical and chemical parameters were monitored during the entire experimental period the degree of virulence was different among the strains. The reasons for this variation are not clear. In line with the degree of virulence stated by [12], *A. veronii* from the skin and *Pseudomonas aeruginosa* from the Kidney

were highly virulent whereas *A. hydrophila subsp dhakensis* from the muscle tissue, *A.veronii* from the kidney and *A. hydrophila* from the intestine were moderately virulent. Previous study on virulence conducted in Bangladesh [5] revealed that *Pseudomonas* and *Aeromonas* caused high mortality up to 50% in the experimental fish using intramuscular injection method at a bacterial challenge dose of 3-6x10^6 CFU/ml. Another study in the Philippines [18] reported *Aeromonas hydrophila* causing cumulative mortality of 50% in the experimental fish. This is lower level of virulence compared to the present study. In summary antibiotic susceptibility assays revealed that 57.66% of the bacterial strains tested were susceptible to the chemotherapeutic agents used although there was individual cases were the strains were almost resistant to more than 80 to 90 % of the tested drugs (Table 2). This result suggests that antibiotics could be employed to prevent outbreaks of diseases particularly in confined environments but not always can eliminate most of strains as some virulent bacteria have developed resistance to most of the chemotherapeutic agents. Efforts are needed to control the disease from occurring rather than treating the disease which is most of the time risky and expensive. In this study, Aeromonas hydrophila was sensitive to Erythromycin, Neomycin, Chloramphenicol,Trimethoprim-sulfamethoxazole and Kanamycin. In contrast, *A. hydrophila* strains isolated from fish in Malaysia were reported to be resistance to most of the drugs used in this experiment [19]. The observed differences in the frequency of resistance might be due to the source of *Aeromonas* isolates and the frequency and type of antimicrobial agents prescribed for the treatment of *Aeromonas* infections in different geographic areas [19]. *Aeromonas veronii* have recently been reported to cause acute death of Channel Catfish (Ictalurus lunetas) in China with severe ascites, extensive hemorrhage on the body surface and organs [20]. Similar clinical characteristics were observed in this experiment. A. hydrophila is described as the dominant infectious agent of 'fish-bacterial-septicemia' in freshwater cultured finfish in China [21] *Aeromonas hydrophila* is also associated with EUS, which is a major problem in Southeast Asia, [22].

Fish diseases caused by *Aeromonas* and *Pseudomonas* have been considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced reduction and low quality of aquatic organisms. Both *Aeromonas spp.* (*A. hydrophila*, *A. sobria*

and *A. caviae*) and *Pseudomonas spp*. (*P.fluorescens*, *P. putida* and *P. aeruginosa*) were incorporated in severe outbreaks among *Anabas sp* in fish hatcheries [2].

The findings from this study are in agreement with several studies conducted elsewhere in which Pseudomonas and Aeromonas were studied for virulence [1, 2, 6, 18, 23-25] . The findings from this experiment can be used to simulate the actual situation happening in the wildness during the disease outbreaks. It is the first extensive study in fish diseases in Lal Dighi where Aeromonas and Pseudomonas are reported as the causative agent of fish infections. The findings also indicated some of the infected fish to develop blindness of the eyes and trachoma; although there are several reports on fish blindness due bacterial infections; no specific bacterial have been mentioned to cause the blindness. In this experiment the re-isolated bacteria from the fishes that developed blindness were pseudomonas, however, the study was not conclusive of whether pseudomonas was the real causative agent of the problem or not.

Clinical abnormalities developed by the experimental fish have been of important in fisheries management; for example, fin erosion has become a concern in fisheries management because of aesthetic and fish survival issues [6]. The erosion of the fins in fish is reported to be caused by several factors including abrasion with rough surfaces, fin damage from aggressive encounters between fish, nutritional deficiencies, and bacterial infection. However from this study the clinical observations after the bacterial treatment are to a larger extent resulting from the bacterial infections as other survival conditions were monitored.

CONCLUSION

The information has provided an important understanding on some of the most pathogenic bacterial strains and their virulence for potential baterial fish pathogens in Midnapur .The study will help in controlling and treating the incidents of bacterial infections in aquaculture ventures as well as in capture fisheries as well as *Anabas sp.*

This knowledge will be of significant to fish farmers in control of fish diseases for improvement of fish productions and ultimately reflects the socio-economic conditions of the farmers and nation as a whole .

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