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Virulence and Drug Sensitivity of *Flavobacterium columnare*, the Causative Agent of Columnaris Disease

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Abstract: Virulence and drug sensitivity of six *Flavobacterium columnare* isolates recovered from different fish species of Bangladesh were compared. Immersion method was employed to infect climbing perch (*Anabus testudineus*) using three different concentrations of bacteria viz. 1x 10^8, 1x 10^7 and 1x 10^6 colony forming units (cfu)/ml. The percentage infection and mortality caused by each isolate varied from 0-100% indicating wide variations in virulence. Isolates F_{03} and F_{32} appeared highly virulent in all challenge doses. At the highest challenge dose of 1x 10^8 cfu/ml, these two isolates were able to kill 100% fish by the end of the trial at day 7 post-challenge. While they killed only 10 and 30% fish respectively, at the lowest dose of 1x 10^6 cfu/ml during this period. Infection in fish due to challenge with these isolates started to appear by day 3 post-challenge. Isolates F_{10} and F_{35} were considered as medium virulent since they were able to kill 50 and 60%, and 20 and 40% fish at 1x 10^8 and 1x10^7 cfu/ml respectively, at the end of the trial. These two isolates were unable either to infect or kill any fish at 1x 10^6 cfu/ml. The other two isolates F_{16} and $F_{25,}$ appeared low or non-virulent. All the six isolates were found sensitive to six selected antibiotic substances appart from isolate F_{16}, which was only sensitive to sulphaurethoxazole.

Key words: Virulence, drug sensitivity, *Flavobacterium columnare*

Introduction

Flavobacterium columnare (Bernardet *et al.,* 1996) (formerly known as *Cytophaga columnaris* or *Flexibacter columnaris*) is the causative agent of columnaris disease which has long been recognized as a pathogen of many freshwater fish species world-wide (Austin and Austin, 1987). Columnaris disease is characterized by gill necrosis, grayish-white or yellow erosive lesion on the body, skin erosion and fin rot. Skin scraping or gill squash preparations from fish with cutaneous columnaries reveal large numbers of long, rod-shaped bacteria arranged in column (Plumb, 1994). The disease is favoured by elevated organic loads, crowded conditions and excessive handling. The bacterium is Gram-negative, exhibits gliding movement, strictly aerobe, actively proteolytic and does not utilize carbohydrate (Wakabayashi, 1993). The mechanisms by which *F. columnare* causes diseases in fish are largely unknown. There is very little information available concerning the actual factors affecting the virulence of the bacterium. Only few studies have attempted to evaluate the first step of the virulence of *F. columnare* (Chowdhury and Wakabayashi, 1988, 1989; Decostere *et al.,* 1999). In Bangladesh, *F. columnare* has recently been isolated from diseased fish of different parts of the country (Sarker *et al.*, 1999). This study is one of the parts of the research on columnaries diseases in Bangladesh Fisheries which is focused on the comparison of the virulence and antibiotic sensitivity pattern of *F. columnare* strains recovered from diseased fish of Bangladesh.

Materials and Methods

Culture and preparation of bacterial isolates: Six *F. columnare* isolates originating from fish of Bangladesh were included in the present study (Table 1). The bacteria were previously characterized by Sarker *et al.* (1999). The bacteria were cultured in Cytophaga broth (Anacker and Ordal, 1959) at 25°C. Bacterial cells were harvested from the broth culture by centrifugation at 5,500 x g for 20 min. The bacterial cells were washed twice with autoclaved formulated water containing 0.03% NaCl, 0.01% KCl, 0.002% $CaCl_2$. $2H_2O$ and 0.004% $MgCl_2$. $6H_2O$ and kept ready for the challenge experiment.

Table 1: Isolates of *Flavobacterium columnare* used in this study

Isolates	Host fish	Year of isolation	Location
F_{03}	*Anabus testudineus*	1999	Eastern part of Bangladesh
F_{10}	*Anabus testudineus*	1999	Eastern part of Bangladesh
F_{16}	*Anabus testudineus*	1999	Eastern part of Bangladesh
F_{25}	*Clarius bactrachu*	1999	Western part of Bangladesh
F_{32}	*Clarius bactrachu*	1999	Middle part of Bangladesh
F_{35}	*Cirrhina mrigala*	1999	Northern part of Bangladesh

Fish: Climbing perch (*Anabus testudineus*) was used to compare virulence of *F. columnare* isolates. Healthy fish of average weight of 20 g were obtained from a rearing pond of the Faculty of Fisheries, Bangladesh Agricultural University and acclimatized in aquarium with aeration for four days in laboratory before use in challenge test.

Water: Above mentioned formulated water which support the long-term survival and high infectivity of *F. columnare* (Chowdhury and Wakabayashi, 1988), was used in present study.

Laboratory challenge: The challenge experiment was conducted at the wet laboratory of the Faculty of Fisheries, Bangladesh Agricultural University. A total of 42 glass tanks having 40 litre capacity were used. Each tank was aerated by aerator and maintained at 25°C.

Immersion method was used to infect fish with six different isolates of *F. columnare* (Table 1) and for each isolate three different concentrations of bacteria viz. 1x 10^8, 1x 10^7 and 1x 10^6 cfu/ml were used. The doses of bacteria and the exposure time were selected on the basis of preliminary infection experiments. Required quantities of each bacterial cells were suspended in two replicated glass tanks (each having 10 litre formulated water) for each challenge dose. Ten fishes were

immersed in each tank containing bacterial suspension. Two control groups 10 fish for each challenge dose were held in formulated water containing no bacteria.

After 24 h post-challenge, 80% of the water of each tank was replaced with formulated water and from the following day, half of the tank water was exchanged daily. Fish was monitored for 7 days. No feed was supplied during the experimental period. The infection was diagnozed by the appearance of external lesion of gray-white to yellowish colored foci, which enlarged gradually. Mortality of fish was recorded and the dead fish were immediately removed from the aquaria. *F. columnare* was reisolated from the dead fish, or if survived, at the end of experimental period. The identification of reisolated pathogen was confirmed according to Chowdhury and Wakabayashi (1988).

Drug sensitivity test: Commonly used discs of six antibiotic compounds were used for the sensitivity test of the selected *F. columnare* isolates (Table 1). The drug discs (Oxoid Ltd.) consists of chloramphenicol (30 µg/disc), oxytetracycline (30 µg/disc), sulphamethoxazole (25 µg/disc), erythromycin (10 µg/disc), streptomycin (10 µg/disc) and oxolinic acid (2 µg/disc). A suspension of the individual isolates cultured 24 h, was prepared in sterile saline and spreaded over the iso-sensi Test Agar (Oxoid) plate. After inoculating over night, the antibiotic discs were dispensed uniformly with the help of oxoid unipath disc dispenser merk 11 and incubated at 20°C. Results of the drug sensitivity was recorded after 48 h of inoculation and expressed as resistant (R), when the growth was normal, or sensitive with the zone of inhibition (measured in mm) having no growth around the drug disc. The zone of confusing growth around the disc was noted as ± .

Results

Variations were found in the virulence of different *F. coulmnare* isolates examined here. The infection and mortality obtained with different isolates varied from 0-100%. The higher challenge doses of 1x 10^8 and 1x 10^7 cfu/ml were able to infect fish successfully (Tables 2 and 3), while only highly pathogenic isolates were able to infect fish at the lowest dose of 1x 10^6 cfu/ml (Table 4). When the fish were exposed to 1x 10^8 cfu/ml of bacterial suspension, lesion started to develop from day 3 post-challenge and a maximum of 70 and 100% fish were infected by isolates F_{03} and F_{32}, respectively. The mortality caused by these two isolates at the same time was 30 and 50% respectively (Table 2). The infection became gradually severe and at the termination of the experiment at day 7 post-challenge, 100% fish got infection and all of them died. Fish exposed to isolates F_{10} and F_{35} at this dose, 70 and 100 % got lesion and 50 and 100% of them died at the end of the trial. Isolate F_{16} was found less pathogenic to fish although it was able to infect only 40% of the fish but was unable to kill any fish at this challenge dose. No fish was affected in the control groups. At the lower dose of 1x 10^7 cfu/ml, 50 and 90% fish were infected where 30 and 50% of them died within 3 days of exposure by isolates F_{03} and F_{32}, respectively (Table 3). The infection and mortality rate gradually increased with time and at the end of the challenge 70 and 100% fishes were killed by these two isolates. The next pathogenic isolate at this dose

Table 2: Infectivity of climbing perch following immersion challenge with different *Flavobacterium columnare* isolates at a dose of 1x 10^8 cfu/ml

Isolates	Cumulative infection and mortality (%)						Re-isolation of *F. columnare*
	Day 3 post-exposure		Day 5 post-exposure		Day 7 post-exposure		
	Infection (%)	Mortality (%)	Infection (%)	Mortality (%)	Infection (%)	Mortality (%)	
F_{03}	70	40	100	80	100	100	+
F_{10}	30	10	50	30	70	50	+
F_{16}	0	0	10	0	40	0	+
F_{25}	40	20	60	20	60	30	+
F_{32}	100	50	100	100	100	100	+
F_{35}	50	30	70	40	100	60	+
Control	0	0	0	0	0	0	ND

ND: Not detected

Table 3: Infectivity of climbing perch following immersion challenge with different *Flavobacterium columnare* isolates at a dose of 1x 10^7 cfu/ml.

Isolates	Cumulative infection and mortality (%)						Re-isolation of *F. columnare*
	Day 3 post-exposure		Day 5 post-exposure		Day 7 post-exposure		
	Infection	Mortality	Infection	Mortality	Infection	Mortality	
F_{03}	50	30	80	40	90	70	+
F_{10}	10	0	40	20	50	20	+
F_{16}	0	0	0	0	0	0	ND
F_{25}	0	0	30	10	40	10	+
F_{32}	90	50	100	70	100	100	+
F_{35}	20	0	50	20	60	40	+
Control	0	0	0	0	0	0	ND

ND: Not detected

Table 4: Infectivity of climbing perch following immersion challenge with different *Flavobacterium columnare* isolates at a dose of 1x 10^6 cfu/ml.

Isolates	Cumulative infection and mortality (%)						Reisolation of *F. columnare*
	Day 3 post-exposure		Day 5 post-exposure		Day 7 post-exposure		
	Infection	Mortality	Infection	Mortality	Infection	Mortality	
F_{03}	5	0	20	10	40	10	+
F_{10}	0	0	10	0	20	0	+
F_{16}	0	0	0	0	0	0	ND
F_{25}	0	0	0	0	0	0	ND
F_{32}	30	10	40	20	60	30	+
F_{35}	0	0	10	0	20	0	+
Control	0	0	0	0	0	0	ND

ND: Not detected

Table 5: Sensitivity patterns of different isolates of *Flavobacterium colamuarre* to different antibiotics.

Isolates	Response to different antibiotic with zone of inhibition (mm)					
	C	OT	SXT	E	S	OA
F_{03}	27	21	11	27	12	19
F_{10}	28	23	15	26	16	25
F_{16}	22	18	R	25	18	19
F_{25}	24	21	8	24	± 15	17
F_{32}	35	26	16	32	25	29
F_{35}	32	27	± 12	29	22	24

C: Chloramphenicol (30 µg/disc), OT : Oxytetracycline (30 µg/disc),
E: Erythromycin (10 µg/disc), SXT: Sulphaurethoxazole (25 µg/disc),
S : Streptomycin (10 µg/disc), OA : Oxolinic acid (2 µg/disc),
R : Resistant, + : Sensitive, ± : Confusing zone

was isolates $F_{35,}$ which was able to infect 60 % fish and only 40% of them died at the end of the trial. The rest of two isolates were found less pathogenic, where isolate F_{16} failed to either infect or kill any fish (Table 3). No death occurred in control groups.

When the challenge dose was further down to 1x 10^5 cfu/ml, the pathogenic activity of all isolates were found to be decreased markedly (Table 4). At this dose however, only isolate F_{32} and F_{03} showed their pathogenic activities to some extent and killed only 30 and 10% fish respectively by the end of the trial (Table 4). The other isolates were unable to kill any fish. No mortality was also recorded in control groups.

It was possible to reisolate *F. columnare* from infected and dead fishes in all cases, and by biophysical and biochemical tests it was confirmed that all infections and mortalities were due to *F. columnare.*

The drug sensitivity patterns of six *F. columnare* isolates are shown in Table 5. All the isolates were sensitive to all the antibiotics used here apart from isolate F_{16}, which was only sensitive to sulphaurethoxazole.

Discussion

Six selected isolates of *F. columnare* recovered from fish of Bangladesh were compared for their ability to produce disease in climbing perch following immersion challenge using three different challenge doses viz. 1x 10^8, 1x 10^7 and 1x 10^6 cfu/ml. The percentage infection and mortality caused by each isolate varied from 0-100% indicating wide variations in virulence. Bacterial virulence is a relative term although it usually implies the ability to cause disease in a host (Sparling, 1983). Virulence factors may act either singly or in combination, at various stages of infection, such as adherence, tissue invasion and systemic dissemination (Dalsgaard, 1993). The cause of the variation in virulence of the isolates of *F. columnare* may be multifactorial and was not studied in present study.

The percent infection and mortality caused by each isolate indicate that there were low, medium and high virulent isolates of *F. columnare* similar to those reported by Morrision *et al.* (1981), Amin *et al.* (1988). Among the six *F. columnare* isolates tested here, isolate F_{32} and F_{03} were found to be highly virulent. Isolate F_{10} and F_{35} were recognized as moderately virulent isolates, and isolates F_{25} and F_{16} were detected as low virulent isolates. Pacha and Ordal (1970) also reported variations in the virulence of *F. columnare* strains recovered from different sampling stations in Colombia River, USA. They observed that epizootic and heavy moralities of fish were caused by highly virulent strain. However, variations in experimental challenge protocols reported by different authors such as the infection route, water temperature and fish size, make it difficult to directly compare the different challenge models used (Paniagua *et al.*, 1990). In the present study climbing perch was used as experimental fish because of its high susceptibility to *F. columnare* infection in Bangladesh. Route of infection was found to be important in determining the disease-producing capacity of bacteria (Pacha and Ordal, 1970). Immersion method was used here to stimulate the natural spread of disease. It was reported that highly virulent *F. columnare* strains were found to produce disease more readily when infection was done by contact than by either intramuscular or intraperitoneal injection (Amin *et al.,* 1988). Again, the experiment was conducted in formulated water since it was found to provide the long-term survival of *F. columnare* (Chowdhury and Wakabayashi, 1988). In the present study the virulence of only six *F. columnare* isolates of Bangladeshi origin were studied and virulence of many more isolates would be compared.

The *F. columnare* isolates appeared to be sensitive to all antibiotics used in this study. Only a few reports are available about the drug sensitive pattern of *F. columnare*. Fijan and Voorhrrs (1969) found that *F. columnare* was sensitive to oxytetracycline, tetracycline and several other drugs. Amed (1970) reported that Sulphanmerazine and oxytetracycline were found effective against *F. columnare* infection when used in feed.

The present study provides a better understanding about the infectivity and sensitivity of different *F. columnare* isolates of Bangladeshi origin which will be helpful for further studies relating the pathogenicity and control measures against *F. columnaris* infection in fish.

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