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Studies on Bacterial Flora in a Farmed Catfish, *Clarias* Hybrid

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Abstract: A ten months investigation was conducted to know the status of bacterial flora in the farmed hybrid catfish (*Clarias* hybrid) and respective pond water from the fish pond of a commercial fish farm of Bangladesh. The average total load in pond water, slime and kidney of fish varied from 2.1×10^3 to 4.1×10^6 CFU/ml of water, 5.6×10^3 to 4.4×10^9 CFU/g of slime and 1.8×10^1 to 2.7×10^4 CFU/g of kidney. Coryneforms and *Flavobacterium* were the dominant bacteria in the observed fish-organs and pond-water. Higher bacterial load was recorded in the months of September and October. Sensitivity of the isolated Aeromonads and Pseudomonads to various antibacterial agents was determined. Higher percentage of resistant Aeromonads and Pseudomonads were observed against oxytetracycline followed by erythromycin. On the contrary, the percentage of resistant Aeromonads and Pseudomonads was lower against oxolinic acid.

Key words: *Clarias* hybrid, Bacterial flora, Fish-disease, Bangladesh

Introduction

Clarias hybrid, a successful artificial crossbreeding of female indigenous catfish (*Clarias batrachus*) and male African catfish (*Clarias gariepinus*) was first produced in Bangladesh by Mollah and Karim (1990). The hybrid is phenotypically similar to *C. batrachus* but the growth rate is comparable to *C. gariepinus*. Some fish farmers have been encouraged to raise this hybrid catfish and the results have been most satisfactory. The market demand is high and the hybrid *Clarias* may be rapidly competing with the African catfish and the indigenous catfish. In farmed condition this hybrid catfish has been reported with lesion, which is suspected as microbial disease. This lesion results an undesirable appearance that affects customer's choice and as well as impacts negatively on market value. Research on fish disease particularly on bacterial disease is scare in Bangladesh, although it is essential for the improvement of fish health (Chowdhury, 1993). Present attempt was made to investigate bacterial disease in a commercial fish farm Bangladesh Fisheries Limited of Mymensingh district of Bangladesh. Present investigation was conducted with a view to study the qualitative and quantitative aspects of bacterial flora in farmed *Clarias* hybrid, determine temporal variation of bacterial load and to study the sensitivity pattern of recovered Aeromonads and Pseudomonads to various antibiotics.

Materials and Methods

Two similar ponds having area of 0.2 hactre and depth 0.2 m each were selected. Culture system was semi-intensive in which emphasis was given on higher stocking density. Sampling was performed from September 1994 to July 1995. Temperature, dissolved oxygen and pH of water of the ponds were recorded throughout the sampling period. Fish samples were taken in polythene bags and water in opaque plastic bottle and carried them immediately Fish Disease Laboratory under the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh.

Culture and isolation of bacteria: For the isolation of bacteria two external organs, the body surface (slime) and the gills were selected considering as usually exposed to the environment. The liver and the kidney were selected as internal organs. The ambient water of the respective ponds was also considered for the same purpose. Tryptone Soya Agar (TSA oxoid) and Plate Count Agar were used for culture and isolation of bacteria. For inoculation of *Aeromonas* spp. and *Pseudomonas* spp., Aeromonas Agar Base and Pseudomonas Agar Base medium were needed respectively. Initially visual inspection of the external body surface was performed to identify any gross external lesions.

The fishes were killed by hurt to the head. The slime from external body surface (including affected surface) was aseptically scrapped with a sterile scalpel and weighted in a sterile test tube and suspended in sterile physiological saline (0.85%) to have a stock solution. In every case desired dilution were made in a physiological saline by ten-fold dilution method. From the dilution sample an amount of 0.1 ml was aseptically pipetted and spreaded over the culture plates and incubated at 25°C for 36-48 hours.

Determination of total number of bacteria: The incubated plates were brought out from the incubator when the colonies were clearly visible. The plates having the colonies within the range from 50-200 were selected for determination of total kind of bacteria and isolation of individual bacterial genera/groups.

$$\text{The total load of bacteria} = \frac{C \times D \times 10 \times V}{W} (\text{CFU/g})$$

where, C = No. of colonies found, D = Dilution factor, V = Volume of physiological saline, W = Weight of the material (kidney/slime).

Characterization of bacteria: Morphological characteristics of the bacterial colonies of the bacteria such as shape, size and color were recorded by eye observation. Shape of the individual isolate was determined by Gram staining method with the young culture. To know the Gram's staining response sometimes 3 percent KOH was also used. The motility test was performed by hanging drop method. Biochemical tests such as catalase activity test, oxidase test, Hugh and Leifson's test, indole production test, gelatine liquefaction test, proteinase test etc. were performed with the fresh culture of bacterial isolates according to the methods described by the Cowan and Steel's Manual for the identification of Medical Bacteria edited by Barrow and Feltham (1993). However the identification of bacterial isolates was confirmed with the help of Bergey's Manual of Systemic Bacteriology (Volume 1 and 2) edited by Krieg and Holt (1984) and Sneath *et al*. (1988).

Confirmation of *Aeromonas* spp. and *Pseudomonas* spp. for antibacterial resistant study: Selective medium, Aeromonas Agar Base (oxoid) supplemented with ampicillin SR 136 and Pseudomonas Agar Base (oxoid) supplemented with C-N supplement (SR 102) were used to confirm Aeromonad isolates and Pseudomonad isolates from pond water and fish organs. Six antibiotics were used to detect the resistant Aeromonads and

Pseudomonads such as oxolinic acid (2 μg/disc), potentiated sulphonomides (25 μg/disc), chloramphenicol (30 μg/disc), streptomycin (10 μg/disc), oxytetracycline (30 μg/disc) and erythromicin (10 μg/disc). A number of 40 Aeromonads and 40 Pseudomonads isolates (20 from pond water, 20 from slime and kidney) were selected to investigate the sensitivity patterns. Suspension of individual isolates was prepared with sterile physiological solution and spreaded over the Iso-Sensi-Test Agar (oxoid). Antibiotic doses were than dispensed over the inoculated plates uniformly with Oxoid Unipath Disc Dispenser Mark-II and inoculated at 25°C for 24 hours. The result of drug sensitivity was determined from the zone of inhibition (measured in mm) around the drug disc on the inoculated culture. The isolates were recognized as resistant when there was no zone of inhibition.

Results and Discussion

The average total bacterial load varied from 2.1×10^3 to 7.1×10^5 CFU/ ml and 2.3×10^3 to 4.1×10^6 CFU/ml of water; 5.6×10^3 to 5.2×10^8 CFU/g and 5.3×10^3 to 4.4×10^9 CFU/g of slime and 1.8×10^1 to 2.7×10^4 CFU/g and 5.6×10^1 to 2.6×10^4 CFU/g of kidney in the pond-1 and pond-2 respectively. Beveridge *et al*. (1991) found 5.4×10^5 to 4.03×10^6 bacterial cells/ml of water in the experimental *Cyprinus carpio* L. cultured tank. Temporal variation of bacterial load in pond water, slime and kidney is shown in Fig. 1. On the basis of the characterization all the bacterial isolates were grouped into 11 general groups. They were tentatively *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Bacillus*, Coryneforms, *Flavobacterium*, *Staphylococcus, Achromobacter*. *Micrococcus*, *Pseudomonas* and *Vibrio*. Austin and McIntosh (1988) isolated *Aeromonas*, *Cytophaga*, *Micrococcus*, *Pseudomonas* and *Staphylococcus* from the eyes and skin of rainbow trout. Lilley *et al*. (1992) found *Pseudomonas*, *Micrococcus*, *Flavobacterium* and Enterobacteria from EUS (Epizootic Ulcerative Syndrome) affected fish. Primary characterization of bacterial isolates recovered from the pond water, slime and kidney is shown in Table 1. Table 2 shows results of the biochemical tests for the bacterial isolates. Higher percentage of resistant *Aeromonads* and *Pseudomonads* were observed against oxytetracycline followed by erythromycin and lower against oxolinic acid.

Table 1: Primary characterization of different isolates

Characteristics	A	B	C	D	E	F	G	H	I	J	K
Gram stain	-	+	-	+	-	-	-	-	+	-	-
Shape	R	R	R	R	S	R	S/R	R	R	R	R
Motility	+	-	-	-	+	+	-	+/-	+/-	-	-
Growth in air	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	-	+	-	-	-	+	+/-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	+	+	+	+	-	-	+/-	+	+
Glucose (acid)	+	+	+	+	+	+/-	+	+	+/-	-	+
Carbohydrates (F/O/-)	F	F	O	O	O	O/-	O	F	F/O	-	O

A: *Aeromonas* B: Coryneforms C: *Flavobacterium* D: *Staphylococcus* E: *Micrococcus* F: *Pseudomonas*
G: *Acinetobacter* H: Achromobacter I: *Bacillus* J: *Alkaligenes* K: *Vibrio* -: Negative
+: Positive S: Spherical R: Rod O: Oxidative F: Fermentative

Table 2: Results of the biochemical tests for the bacterial isolates

Characteristic	Response by different bacterial genera/groups										
	A	B	C	D	E	F	G	H	I	J	K
Indole production	+	+	+	-	-	+	+	+	-	Nt	+
Gelatin liquefaction	+	-	+	Nt	-	-	-	-	-	Nt	+
Protinase (casin) test	+	+	-	Nt	-	-	-	-	+	Nt	-
Resistant	+	Nt	Nt	Nt	Nt	+	Nt	Nt	Nt	Nt	-

A: *Aeromonas* B: Coryneforms C: *Flavobacterium* D: *Staphylococcus* E: *Micrococcus* F: *Pseudomonas*
G: *Acinetobacter* H: Achromobacter I: *Bacillus* J: *Alkaligenes* K: *Vibrio* -: Negative
+: Positive Nt: Not tested

Table 3. Antibiotic sensitivity patterns of selected *Aeromonas* spp.

Source of Isolate	Strain No.	Sensitivity to antibacterial agents (drug disc) with their zone of inhibition (mm)					
		C	OT	SXT	E	S	OA
Water	1	+18	R	R	+19	R	+31
	2	+19	R	+23	±10	±6	+29
	3	R	R	+27	R	+14	R
	4	±16	+6	R	R	+26	+24
	5	+23	R	+19	R	+19	+26
Fish Organ	1	R	±6	+26	R	±6	+18
	2	+23	R	R	+26	+26	+14
	3	+22	R	±18	±10	+27	+26
	4	+24	R	+22	R	+30	R
	5	+26	+8	+20	R	+21	+32

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

Table 4: Antibiotic sensitivity patterns of selected *Pseudomonas* spp.

Source of Isolate	Strain No.	Sensitivity to antibacterial agents (drug disc) with their zone of inhibition (mm)					
		C	OT	SXT	E	S	OA
Water	1	+20	R	+22	R	R	±12
	2	R	+28	+24	+19	R	+31
	3	R	R	R	R	+19	+29
	4	+26	R	+27	R	+27	+27
	5	+29	R	R	R	+26	+26
Fish Organ	1	+30	R	+19	R	R	R
	2	R	R	R	R	+22	R
	3	R	R	+32	R	R	+32
	4	+22	±12	+28	±18	19	+26
	5	+18	R	+24	R	+20	+28

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

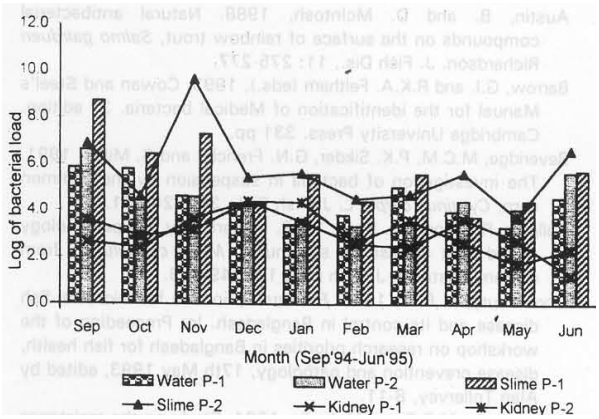

Fig. 1: Temporal variation of bacterial load in water, slime and kidney

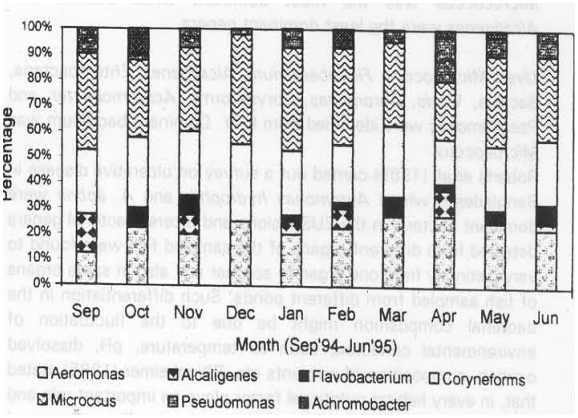

Fig. 3: Temporal prevalence in percentage of bacterial gener/ groups in slime

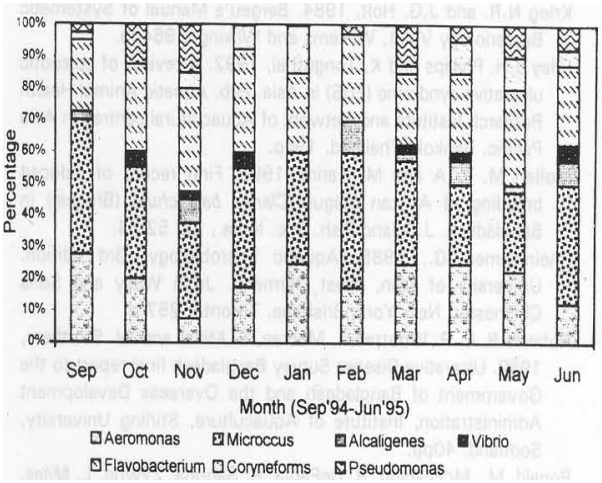

Fig. 2: Temopral prevalence in percentage of bacterial genera/ groups in pond water

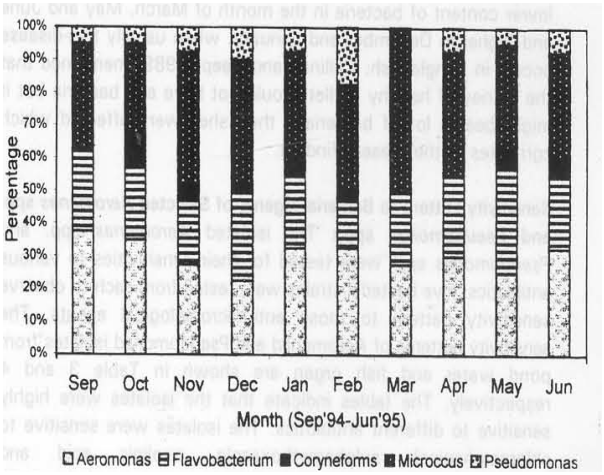

Fig. 4: Temporal prevalence in percentage of bacterial genera grouups in kidney

Identified bacterial genera/groups from pond water and fish organs

Water: From the pond water isolated bacterial genera/groups were *Aeromonas*, *Micrococcus*, *Alcaligenes, Vibrio, Flavobacterium*, *Bacillus, Pseudomonas* and Coryneforms. *Micrococcus* was found to be the most dominant group followed by *Flavobacterium* and *Aeromonas*.

Slime: The isolated bacterial bacterial genera/ groups in the slime were *Aeromonas*, *Alcaligenes, Flavobacterium*, Coryneformes, *Micrococcus*, *Pseudomonas* and *Achromobacter. Micrococcus* was the most dominant followed by Coryneformes. The least

dominant bacterium was *Achromobacter*.

Kidney: In the kidney, isolated bacterial genera/groups were *Aeromonas*, *Flavobacterium*, Coryneformes, *Micrococcus, Pseudomonas* and *Achromobacter* in which *Micrococcus* was the most dominant followed by *Aeromonas*.

Lesion: Identified bacterial genera/groups in the lesions were *Pseudomonas, Aeromonas, Micrococcus* and *Flavobacterium*. *Aeromonas* was dominant bacterium. In case of pond-1 no lesion was found in the month of September, December, February, March and June.

Gill: *Aeromonas*, *Pseudomonas*, *Micrococcus*, *Flavobacterium*, *Bacillus*, *Vibrio, Achromobacter, Staphylococcus, Alcaligenes* and Coryneforms were detected from the gill of sampled fish. *Micrococcus* was the most dominant while *Bacillus* and *Alcaligenes* were the least dominant genera.

Liver: *Micrococcus*, *Flavobacterium*, *Alcaligenes,* Enterobacteria, Bacillus, *Vibrio*, *Aeromonas*, Coryneforms, *Achromobacter* and *Pseudomonas* were identified from liver. Dominant bacterium was *Micrococcus*.

Roberts *et al*. (1989) carried out a survey on ulcerative disease in Bangladesh, where *Aeromonas hydrophila* and *A. sobria* were dominant bacteria in the EUS lesions and ulcers. Bacterial genera detected from different organs of the sampled fish were found to vary distinctly from one organ to another and also in same organs of fish sampled from different ponds (Fig. 1). Such differentiation in the bacterial composition might be due to the fluctuation of environmental condition, such as temperature, pH, dissolved oxygen, composition of nutrients etc. Rheinheimer (1985) stated that, in every habitat nutritional factor plays an important role and influences decisively the composition of microflora. Temporal prevalence in the percentage of bacterial genera/groups in water, slime and kidney are shown in Fig. 2, 3 and 4 respectively. The kidneys of sampled fishes were observed to have lower content of bacteria in the month of March, May and June and higher in December and January, when usually fish-disease occurs in Bangladesh. Callinan and Keep (1989) mentioned that the kidney of healthy mullets could hot have any bacteria but it might bear a lot of bacteria if the fishes were affected which correlates to the present finding.

Sensitivity Pattern to Bacterial Agents of Selected *Aeromonas* spp. and *Pseudomonas* spp.: The isolated *Aeromonas* spp. and *Pseudomonas* spp. were tested for their sensitivities to various antibiotics. Five bacterial strains were tested from each to observe sensitivity pattern to those anti-microbiological agents. The sensitivity patterns of Aeromonad and Pseudomonad isolates from pond water and fish organ are shown in Table 3 and 4 respectively. The tables indicate that the isolates were highly sensitive to different antibiotics. The isolates were sensitive to chloramphenicol, sulphamethoxazole, oxolinic acid and streptomycin. The tendency of resistance patterns both of the two types of isolates recovered from two ponds was more or less same. The highest percentage of resistance was observed against oxytetracycline and erythromycin. Many of the isolates showed multiple patterns of resistance which perhaps due to use of antibiotics and other chemicals indiscriminately to cure EUS and other related disease.

According to McPhearson *et al.* (1991) the aquaculture management practices like stocking densities, chemical treatments etc. might effect antibiotic resistant. Chowdhury and Inglis (1994) observed that the use of antibacterial agents in aquaculture to protect the bacterial disease of fish had caused increase in drug resistant bacteria.

It was not possible to detect the specific pathogenic strain but it was suspected from the symptoms exhibited by the fish investigated that Aeromonads and Pseudomonads could be one of the causative agents. The major bacteria recovered from the lesion were *Aeromonas* spp. and *Pseudomonas* spp. This result supported the suspicion of those fish pathogen. With the preserved isolates, further study is recommended to know their pathogenicity and to find out the appropriate control measures against the disease caused by the fish pathogen.

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