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Bacterial Flora of Koi (*Anabas testudineus*) Harvested from Ponds and Their Antibiogram

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A B S T R A C T

Fish is known to harbour bacteria of public health importance. Aquatic environments of ponds are known to influence the bacterial loads of the harvested fish. The present work was undertaken to determine total viable count (TVC) and isolation and identification of bacteria from Koi fish, mud and water samples of two selected ponds managed and owned by the Bangladesh Fisheries Research Institute (BFRI) and two ponds located at Muktagacha, Mymensingh, managed by private owners. Fish (n=16), mud (n=16) and water (n=16) samples were collected from all 4 ponds. Samples were cultured on plate count agar to determine TVC. Fish harvested from Muktagacha ponds had statistically higher bacterial count (8.44 ± 0.04 $\log_{10}$ CFU/ml) when compared to BFRI ponds (7.92±0.17 $\log_{10}$ CFU/ml) ($p \leq 0.05$). Similarly, highest TVC was found in mud and water samples of Muktagacha ponds (6.87±0.73 and 7.41±0.04 $\log_{10}$ CFU/ml, respectively) compared to mud and water samples of BFRI ponds (5.04 ±0.07 and 5.40±0.09 $\log_{10}$ CFU/ml, respectively). Samples were inoculated onto appropriate selective media for isolation of bacteria. Total 257 bacterial isolates representing five genera were identified: *Pseudomonas* spp. (21.40%), *Aeromonas* spp. (33.46%), *Vibrio* spp. (14.78%), *Salmonella* spp. (21.40%) and *E. coli* (8.94%). Antibiotic sensitivity assay showed multidrug resistant profiles of *Pseudomonas* sp., *Aeromonas* sp., *Salmonella* sp. and *E. coli*. These bacteria are known to cause food borne illness in humans and spoilage of fish.

Keywords: Total viable count, Koi fish, Antibiotic sensitivity assay, multidrug resistant bacteria

Introduction

Climbing perch commonly known as Koi (*Anabas testudineus*) in Bangladesh is a fresh water fish found in small rivers, canal and swamp (Mijkherjee *et al.,* 2002). It is one of the delicious fish with high market demand in Bangladesh (Hossain *et al.,* 2012a). More recently, commercial Koi fish farming in pond became very popular. Many studies have shown that bacteria belonging mostly to the genera *Aeromonas, Corynebacterium, Myxobacterium, Pseudomonas* and *Vibrio* cause infectious diseases in fish (Roberts, 1978). *Aeromonas* spp*., pseudomonas* spp. almost invariably present in every Koi fish pond cause life threatening bacterial infection such as ulcer, fin rot and tail rot (Rahman *et al.,* 2010) in Koi fish. Bacterial load in ponds is very important aspects of water quality. Poor water quality cause burn off the slime coat or stress the koi making it more susceptible to the diseases caused by bacteria (Svobodova *et al.,* 2003; Bonga, 1997).

Fish is involved in active and passive transfer of a wide range of bacterial infection and intoxication to humans caused by *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Aeromonas* spp. (Gold and Salit, 1993) underscoring the need to investigate public health risk associated with fish. Prophylactic use of antibiotics and growth promoters in intensive fish feed is responsible for development of antibiotic resistance in consumers (World Health Organization, 1999), which is an important public health issue in the recent time. The objectives of this study were (i) to determine bacterial loads and identify bacteria from pond-raised Koi and (ii) to determine their antibiotic resistant profiles.

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Materials and Methods

Collection of samples

Fish (n=16), mud (n=16) and water (n=16) samples were aseptically collected from BFRI and Muktagacha ponds from January to June 2013. Fishes were caught by using a net. A total of 48 tissue samples comprised of skin (n=16), gills (n=16) and intestine (n=16) were aseptically collected from 16 fish. The average body weight of fish of BFRI ponds was 65±10.80 g and Muktagacha ponds was 73.75±2.5 g. Samples were collected between 10.00 and 10.30 am at monthly interval during March, April and May 2013. Fish, mud and water samples were placed in sterile plastic bags, petridishes and test tubes, respectively. Samples were transferred to an ice box and transported directly to the Bacteriological laboratory of the Department of Microbiology and Hygiene for microbiological analysis.

Total viable count

Skin, gill and intestine of fish samples were minced and grinded together in 1% peptone water. 0.5 ml of fish, mud and water samples were transfered to 4.5 ml of 1% peptone and a 10-fold serial dilutions were prepared upto 10^{-8}. One ml of each of 10-

fold diluted sample was spreaded onto plate count agar and incubated at 37°C for 24-48 hrs. The mean number of colony (30-300) of three plates in a paticular dilution was multiplied by the dilution factor to determine TVC which was expressed as mean logarithm of the number of colony forming units (log_{10}CFU/ml).

Isolation of bacteria

Skin, gills and intestine contents of fish, mud and water samples were enriched seperately in the nutrient broth at 37 °C for 24 hrs. The overnight enriched broth was streaked onto various selective media such as: Salmonella-Shigella (SS) agar (for *Salmonella* spp.*)*, Eosine methylene blue (EMB) agar (for *E. coli*), M-Aeromonas (MA) agar (for *Aeromonas* spp.), Acetamide agar (AC) agar (for *Pseudomonas* spp.), Thiosulfate citrate bile salt sucrose (TCBS) agar (for *Vibrio* spp.) and incubated at 37 °C for 24 hrs. Single colony was further subcultured until pure culture of bacteria was obtained.

Identification of bacteria

Colony characteristics of bacteria such as: shape, size, surface texture, edge, elevation and colour observed in pure culture, Gram's staining and biochemical tests (Sugar fermentation, Methyl red, Voges-Proskauer and Indole production tests) were used for identification of bacteria (Cheesbrough, 1985).

Antibiotic sensitivity test

One isolate randomly selected from five genera were tested for antimicrobial drug susceptibility against five commonly used antibiotics such as: ampicillin (10 µg/disc), chloramphenicol (30 µg/disc**)**, ciprofloxacin (5 µg/disc**)**, gentamicin (10 µg/disc) and cefalexin (30 µg/disc) by disk diffusion or Kirby-Bauer method (Bauer *et al*., 1966). Results of antibiotic sensitivity tests were recorded as sensitive, intermediate and resistant according to the guidelines of Clinical Laboratory Standard Institute (CLSI, 2007).

Statistical analysis

Total viable count (TVC) of fish, mud and water samples of BFRI and Muktagacha ponds were analyzed for statistical significance using student's t test. A p value of ≤ 0.05 was considered to be statistically significant.

Results

Total viable count of bacteria

In March, TVC of fish, mud and water samples of BFRI ponds were 7.916±0.165, 6.566±0.0759 and 5.172±0.196 and Muktagacha ponds were 8.438±0.035, 7.367±0.021 and 7.308 ±0.216 log_{10}CFU/ml, respectively (Fig.1).

In April, TVC of fish, mud and water samples of BFRI ponds were 8.050±0.019, 5.042±0.066 and 5.398 ±0.085, and Muktagacha ponds were 8.434 ±0.034, 6.874±0.733 and 7.413±0.039, log_{10}CFU/ml, respectively (Fig. 2).

In May, the TVC of fish, mud and water samples of BFRI ponds were 8.361±0.021, 6.611 ±0.747 and 5.377 ±0.105, and Muktagacha ponds were 8.407±0.051, 7.342 ±0.025, and 7.409±0.009 log_{10}CFU/ml, respectively (Fig. 3). The TVC of fish, mud and water were higher in Muktagacha ponds as compared to BFRI ponds in all occasions ($p< 0.05$).

Isolation and identification of bacteria

Five genera of bacteria were identified: *Pseudomonas* spp*., Aeromonas* spp., *Vibrio* spp., *Salmonella* spp. and *E. coli*. The bacteria were isolated from the skin, gill and intestine of Koi, mud and water samples (Table 1). The bacterial idenfication were confirmed by the cultural characteristics, Gram staining, sugar fermentation and biochemical tests.

Antibiotic sensitivity tests

Pseudomonas sp., *Aeromonas* sp., *Salmonella* sp. and *E. coli* were resistant against two different antibiotics, and *Vibrio* sp. was found to be resistant to only one antibiotic (Table 2).

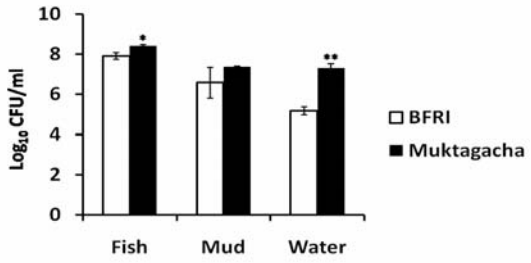

Fig. 1. Total viable count of bacteria in fish, mud and water samples of BFRI and Muktagacha ponds determined in the month of March 2013. The results are expressed as mean log CFU±SD. Statistically significant difference of total viable count of samples (fish, mud and water) between BFRI and Muktagacha ponds are indicated by asterisks(*, $p<0.05$; **, $p<0.001$).

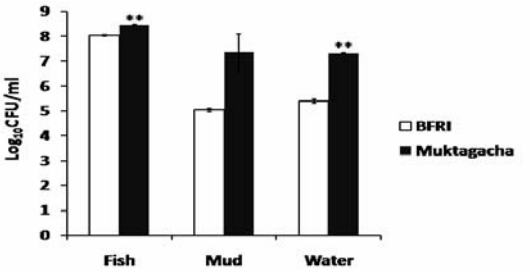

Fig. 2. Total viable count of bacteria in fish, mud and water samples of BFRI and Muktagacha ponds determined in the month of April 2013. The results are expressed as mean log CFU±SD. Statistically significant difference of total viable count of samples (fish, mud and water) between BFRI and Muktagacha ponds are indicated by asterisks(*, $p<0.05$; **, $p<0.001$).

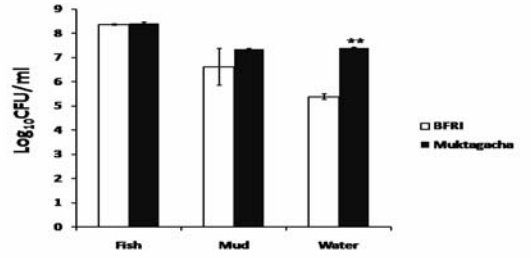

Fig. 3. Total viable count of bacteria in fish, mud and water samples of BFRI and Muktagacha ponds determined in the month of May 2013. The results are expressed as mean log CFU±SD. Statistically significant difference of total viable count of samples (fish, mud and water) between BFRI and Muktagacha ponds are indicated by asterisks (**, $p<0.001$).

Discussion

The ponds and rivers that harbour fish serve as sources of bacterial contamination due to indiscriminate deposition of human and animal excreta as well as other environmental wastes into natural water (Nemec and Massengale, 2010). Free roaming animals and pets especially dogs also contribute to faecal contamination of surface water. Septic system failure, run-off from roads, parking lots and yards can carry animal wastes into natural water and ponds (Ahmed *et al.,* 2005). Birds can also be a significant source of bacteria. Swans, Geese and other water fowl can also elevate bacteria counts in water bodies and ponds (Doyle and Ericson, 2006).

This study recorded statistically significant higher TVC in fish harvested from Muktagacha ponds as compared to fish caught

Table 1. Summary of isolated bacteria

Name of samples (n)	Name of ponds	Bacterial genera				
		***Pseudomonas* spp. (n)**	***Aeromonas* spp. (n)**	***Vibrio* spp. (n)**	***Salmonella* spp.(n)**	***E. coli* (n)**
Skin (16)	BFRI pond	2	3	2	2	ND
	Muktagacha pond	4	9	3	7	ND
Gill (16)	BFRI pond	9	11	ND	2	ND
	Muktagacha pond	9	13	6	6	3
Intestine (16)	BFRI pond	6	8	6	3	ND
	Muktagacha pond	16	21	10	11	6
Mud (16)	BFRI pond	ND	ND	ND	3	ND
	Muktagacha pond	7	9	6	8	4
Water (16)	BFRI pond	ND	ND	ND	5	5
	Muktagacha pond	2	12	5	8	5
Total		55	86	38	55	23

ND = Not detected; Bangladesh Fisheries Research Institute

Table 2. Antimicrobial sensitivity assay of *Pseudomonas* sp., *Aeromonas* sp., *Vibrio* sp., *Salmonella* sp. and *E. coli*

Name of bacteria	Antibiotic sensitivity profiles of isolates				
	Ciprofloxacin	Chloramphenicol	Gentamicin	Ampicillin	Cefalexin
Pseudomonas sp.	S	S	S	R	R
Aeromonas sp.	S	S	S	R	R
Vibrio sp.	I	S	S	I	R
Salmonella sp.	S	S	S	R	R
E. coli	S	S	S	R	R

R=Resistance; S=Sensitive; I=Intermediate

from BFRI ponds ($p<0.048$). In case of mud, the TVC was higher in the Muktagacha ponds when compared to BFRI ponds which was not statistically significant ($p<0.274$). In case of water, the higher TVC was recorded in Muktagacha ponds in comparison to BFRI ($p<0.001$). Tiamiyu *et al*. (2011) reported 6.09±0.65 Log CFU microbial load in skin sample of fish. In this study higher TVC of fish was recorded in all occasions.

The permissible count of heterotrophic bacteria in the $1cm^2$ of skin ranges from $10^2 – 10^7$ or bacteria of $\log_{10}$ CFU/$cm^2 \leq 5.70$ according to International Commission on the Microbiology Specification of Foods (Zmysowska *et al.*, 2000). This study recorded higher TVC in fish than the recommended value. The higher TVC of fish in this study might be resulted from poor water quality, consumption of bacteria by the fish for long time through food and water. The survival of these bacteria in ponds is dependent on the conditions prevailing in the aquatic environment and fish are often simply their hosts (Zmysowska *et al.*, 2000).

In this study, five genera of bacteria, such as *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp., *Salmonella* spp. and *E. coli* were isolated from fish, mud and water samples from the designated locations. The results of isolation of bacteria are in agreement with the findings of Adebayo-Tayo *et al.* (2012) and Truong *et al.* (2008).

In the present study, the colony and Gram's staining characteristics of *Pseudomonas* spp., *Vibrio* spp., *Salmonella* spp. and *E. coli* were similar to the findings reported by many investigators (Thomas *et al.,* 1998; Sharada *et al.,* 1999; Hossain, 2002; Izumi *et al.,* 2004; Samad, 2005; Khan *et al.,* 2007; Faruque *et al.,* 2008).

In this study *Pseudomonas* sp. was found sensitive to chloramphenicol, gentamycin and ciprofloxacin and resistant to cefalexin and ampicillin. *In vitro* antibiotic sensitivity test of the *P. fluorescens* isolates were conducted by disk diffusion method against seven antibiotics where, all of the isolates were found to be sensitive to only against streptomycin and gentamycin but, most of the isolates (80%) were found resistant to chloramphenicol (Foysal *et al.,* 2011). As regards to *Aeromonas* sp., it was sensitive to ciprofloxacin, chloramphenicol and gentamicin and resistant to cefalexin and ampicillin. Truong *et al.* (2008) found two isolates of *Aeromonas hydrophila* were sensitive to sulphamethoxazole and ciprofloxacin.

In this study *Vibrio* sp. was sensitive to chloramphenicol, gentamycin, and intermediately sensitive to ampicillin, ciprofloxacin and resistant to cefalexin. Almost similar antibiogram profiles were also reported by Hossain *et al*. (2012). As regards to *Salmonella* sp., this was sensitive to chloramphenicol, gentamycin and ciprofloxacin and resistant to cefalexin and ampicillin. These findings are in agreement with the result of Frech and Schwarz (1998) and Chugh and Suheir (1983). In this present study, *E. coli* isolate of fish was sensitive to chloramphenicol, gentamycin and ciprofloxacin and resistant to cefalexin and ampicillin. These findings were almost similar to the findings of other researchers (Hasina, 2006; Rahman *et al*., 2008).

Identification of bacteria at species and below species level is often vital to differentiate pathogenic and non-pathogenic forms. The current study could not identify bacteria at species level and differentiate the pathotype of bacteria due to lack of use of modern tests. Bacteriological screening of small number of samples was a limitation of the current study.

Conclusions

Data of this study suggest that Koi harbours multidrug resistant bacteria and TVC of Koi fish was found to be higher than the recommended value which are important issues from the public health point of view. In order to protect public health farmers should embrace standard operating practices as applicable to fish farming. People working in the fish farms should be educated and well trained on the maintenance of good hygienic practices including regular disinfection of catching gears or working equipment, and brief immersion of caught fishes in brine water to reduce the microbial load on the fish before storing at cold temperature or sold to the public.

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