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Bacterial community structure and infection in cultured *Koi* (*Anabas testudineus*) fish species

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Abstract
The study examined the presence of pathogenic and non-pathogenic bacteria species in cultured *Koi* (*Anabas testudineus*). A total of twelve (12) samples were collected for the examination of bacteria. Pelleted feed with 2 g/kg Oxytetracycline (OTC) was applied in the pond. Tissues from the gill, *intestine* and *skin* were excised under aseptic conditions before and after (OTC) treatment for quantitative analysis. Bacterial population was decreased significantly after OTC treatment but it was significantly increased in control system in skin, gills and intestine of *Koi*. Respective samples were inoculated in Brain Heart Infusion broth (BHI) and incubated then subsequently streaked on MacConkey Agar and Blood Agar for qualitative analysis. Bacteria colony was taken through a series of test for identification. Bacterial species belonging to the genus *Staphylococcus, Pseudomonas, Flavobacterium, Escherichia, Salmonella* and *Vibrio* were isolated. Bacterial infection in fish farm is crucial to the fish and consumer. The presence of these types of pathogens means the outbreak of serious fish disease in Bangladesh.

Keywords: *Koi*, *Anabas testudineus*, oxytetracycline, pathogenic bacteria

Introduction
Aquaculture has become one of the fastest developing source of animal protein for humans and animals due to dwindling wild fish stocks around the world and in particular Bangladesh (Al-Harbi and Uddin, 2005) [2]. It is estimated that fish provides at least 50% of total animal protein intake in some small island developing states like Bangladesh (Smith *et al*., 2010) [19]. *Anabas testudineus* known as *Koi* in Bangladesh found in small rivers, canal and swamp (Mijkherjee *et al*., 2002) [14] and it is a mouth-watering fish with high market value in Bangladesh (Hossain *et al*., 2012) [11]. More recently, commercial *Koi* fish farming in pond became very popular. Fish production is decreasing due to climate changes (Halim *et al*., 2017) [9], low water quality, fish disease and unplanned used of antibiotic in fish farm (Hossain *et al*., 2014) [12]. A number of research works is being carried out on the microbiology of freshwater and marine environment in different parts of the world (Al-Harbi and Uddin, 2005) [2]. Fish takes a large number of bacteria in their gut from water, sediment, and food (Sugita *et al*., 1985) [21]. Al- Harbi (2003) [3] suggested that the bacterial flora on fish reflects the aquatic environment. Due to intensification and commercialization of aquaculture, disease is a major concern in the fish farming Industry which caused by viral, bacterial, fungal or parasites pathogens. Disease poses a serious threat to the fish in terms of their survival and growth rates. It is reported by World Bank that financial losses associated with fish disease were in the range of US$ 3 million per annum (Faruk *et al*., 2004) [7]. Bacterial infection also represents a limiting factor for the further development of aquaculture (Austin and Austin, 2007) [5]. Many studies have shown that bacteria belonging mostly to the genera *Aeromonas*, *Corynebacterium*, *Myxobacterium*, *Pseudomonas* and *Vibrio* cause infectious diseases in fish (Ampofo and Clerk, 2010) [4]. Most dominant diseases causing bacteria are *Aeromonas, Corynebacterium, Myxobacterium, Pseudomonas* and *Vibrio* which are responsible for mass mortalities, reduced production and low quality of aquatic organisms (Roberts, 1978) [18]. *Aeromonas* spp. and *pseudomonas* spp. liable for ulcer, fin rot and tail rot in *Koi* (Rahman *et al*., 2010) [15]. Bacterial species isolated from *Koi* could have serious health issues for humans who consume or ingest them. Some of the bacteria species that are isolated from the gut of fish are fecal coliforms.

Cleaning and evisceration of fish is a common route for pathogenic infection in humans (Adebisi and Emikpe, 2017) [1]. Antibiotics are used as a fish diseases management and also for enhancing the growth and effectiveness of feed conversion during the last decades. The objectives of this research were to determine the qualitative and quantitative analysis of bacterial species in *Anabes testudineus* and which organ(s) was more susceptible to bacterial infection.

Materials and Methods

Four experimental ponds were selected to carry out the present experiment. *Koi* fry were collected from Al-amin fish hatchery, Mymensingh (24°45'14.0"N 90°24'11.0"E) and released in the pond. Cast net were used to collect fish samples from each four ponds. Three *Koi* from each pond were randomly selected and length, weight were measured with measuring board and electronic balance respectively. After which they were placed in plastic bag and transported to the laboratory. The fishes were killed by physical destruction of the brain and the number of incidental organisms was reduced by washing the fish skin with 70% ethanol before opening the ventral surface of the belly with sterile scissors to expose the body cavity. Around 0.5-1 g each of skin, gills and intestinal content were taken aseptically and homogenized by mortar. Approximately 0.2 g of each homogenate was then put in a tube containing 2 ml of sterile saline solution. One milliliter of each homogenate solution was serially diluted (10^{-3} to 10^{-7}). Samplings were done every alternative day after treatment. For qualitative analysis excised *Koi* tissues were placed into Brain Heart Infusion broth (BHI) and then incubated for 24 hrs at 37 ºC. These were inoculated into sterile MacConkey (MA) and Blood Agar (BA) and incubated for 24 hrs at 37 ºC. Bacteria colonies were identified by physical characterization and staining. Pure colonies were then taken through a series of standardized biochemical tests, including Indole, S. Citrate, Urea, Motility, H_2S, Catalase, Oxidase, D-Glucose, D-Mannitol, Dextrose, Inositol, Fructose and Galactose. Bergey's Manual of Determinative Bacteriology (Holt, 1977) [10] was used to analyze the various biochemical reactions in order to identify and classify the bacteria. Statistical paired-sample T test was used to test the mean significant difference of bacterial occurrence among the gill, skin and intestine by using SPSS, the significant level was set at $P<0.05$.

Results

Staphylococcus, S. saprophyticus, Pseudomonas, Aeromonas were present in gill and skin, but absent in intestine. *Enterobacter, Escherichia, Shigella* and *Vibrio* species were present in intestine, but absent in gill and skin (Table 1). Bacterial diversity was abundant in control than treated ponds. Bacterial species belonging to the genus *Staphylococcus, Bacillu, Enterobacter, Pseudomonas, Flavobacterium, Escherichia, Streptobacillus, Aeromonas, Salmonella, Shigella*, and *Vibrio* were isolated from fish ponds (Table 2).

Table 1: Bacterial diversity in the tissues of *Anabas testudineus* from four experimental ponds.

Bacterial species	Pond No. 1 (Treated)						Pond No.2 (Treated)					
	Gill No.	%	Skin No.	%	Intestine No.	%	Gill No.	%	Skin No.	%	Intestine No.	%
Staphylococcus spp.	3	27.27	2	16.67					2	20	1	9.09
S. saprophyticus	2	18.18	3	25			3	37.5	1	10		
Bacillus spp.	1	9.09			1	7.14	2	25	2	20		
Enterobacter cloacae											3	27.27
Pseudomonas spp.	2	18.18	1	8.33	1	7.14			3	30		
Flavobacterium spp.	2	18.18	1	8.33			1	12.5	1	10		
Escherichia coli					3	21.43					1	9.09
Streptobacillus			2	16.67			2	25	1	10		
Aeromonas hydrophila	1	9.10	3	25	2	14.29						
Salmonella typhi											1	9.09
Shigella spp.					3	21.43					2	18.18
Enterobacter aerogenes					2	14.29					1	9.09
Vibrio cholerae					2	14.29					2	18.18
Total	11		12		14		8		10		11	

Bacterial species	Pond No. 3 (Treated)						Pond No.4 (Control)					
	Gill No.	%	Skin No.	%	Intestine No.	%	Gill No.	%	Skin No.	%	Intestine No.	%
Staphylococcus spp.	1	11.11			1	8.33	2	7.69	1	4.35	1	3.33
S. saprophyticus	1	11.11	2	28.57	1	8.33	1	3.85	2	8.69	2	6.67
Bacillus spp.	1	11.11					3	11.54	2	8.69	1	3.33
Enterobacter cloacae					2	16.67			1	4.36	2	6.66
Pseudomonas spp.							2	7.69	2	8.6	1	3.33
Flavobacterium spp.	3	33.33	2	28.57			2	7.69	3	13.04348		
Escherichia coli					3	25					6	20
Streptobacillus	1	11.11	2	28.57			3	11.53	1	4.347826	2	6.666667
Aeromonas hydrophila	2	22.22	1	14.29			2	7.69	2	8.69	1	3.33
Salmonella typhi					1	8.33	3	11.53	2	8.695652	3	10
Shigella spp.					2	16.67	2	7.69	2	8.695652	4	13.3
Enterobacter aerogenes					1	8.33	4	15.38	3	13.04348	2	6.66
Vibrio cholerae					1	8.33	2	7.69	2	8.695652	5	16.6
Total	9		7		12		26		23		30	

Table 2: Biochemical reactions of identified bacterial species.

Species/Test	Gram reaction	Shape	Indole	S. Citrate	Urea	Motility	H_2S	Catalase	Oxidase	D-Glucose	D-Mannitol	Dextrose	Inositol	Fructose	Galactose
Staphylococcus spp.	+	c	-	-	+	+	-	+		+	+	+	-	+	+
S. saprophyticus	+	c	-	-	+	+	-	+							
Bacillus spp.	+	b	-		-	-	+	+	+	+	+		-	+	+
Enterobacter cloacae	-	b	-	+	+	+	-	+	-	+	+	+	+	+	+
Pseudomonas spp.	-	*b*	-	-	-	-	-	*(+)*	+	+	+		-	+	-
Flavobacterium spp.	-	cb	-					+		+	-	+	-	+	+
Escherichia coli	-	b	+	-	-	-	-	+	-	+	+	+	-	+	+
Streptobacillus spp.	-	b	-	-	-	-	-	-		+	-	-	-	+	+
Aeromonas hydrophila	-	b	+	-	-	+	-	+	+	+	+		-		
Salmonella typhi	-	b	-	-	-	+	(+)	+	-	+	+				
Shigella spp.	-	*b*	-	-	-	-	-	-	-		-		-	-	-
Enterobacter	-	b	-	+	-	(-)	-	+	-	+	+	+	+	+	+
Vibrio cholerae	-	curve	+	-		+		(-)	+	+	+				

LEGEND: b = bacillus, + = most strains positive, - = most strains negative, (+) = few positive, (-) = few negative, c = cocci, cb = cocco bacilli.

Changes in bacterial load in skin (cfu/cm²): In the day-0 (before treatment) there was bacterial load in skin sample ($6.05\pm0.15\times10^5$) cfu/cm² in treated while in control was ($6.17\pm0.20\times10^5$) cfu/cm². In the times of using antibiotic, bacterial load of skin sample was increased then decreased significantly (P=0.009). The higher and lower bacterial load of sample found ($7.21\pm0.10\times10^5$) cfu/cm² in the day-1 and ($4.24\pm0.05\times10^4$) cfu/cm² in the day-15 respectively. In the control there was higher bacterial load ($9.52\pm0.10\times10^5$) cfu/cm² in the skin (Table 3).

Changes in bacterial load in Intestine (cfu/g): There was bacterial load ($4.51\pm0.10\times10^7$) cfu/g in treated before treatment while in control was ($4.54\pm0.10\times10^7$) cfu/g. Due to antibiotic treatment there was higher bacterial load in intestine ($5.8\pm0.10\times10^7$) cfu/g and finally significantly (P=0.007) reduced to ($2.93\pm0.10\times10^6$) cfu/g in the day-15. In the control there was higher bacterial load ($8.12\pm0.10\times10^7$) cfu/g in the Intestine (Table 3).

Changes in bacterial load in gill (cfu/g): In the day-0 bacterial load in gill sample was ($8.36\pm0.15\times10^6$) cfu/g in treated while in control was ($8.25\pm0.15\times10^6$) cfu/g. Bacterial load of gill sample was increased to ($8.83\pm0.10\times10^6$) cfu/gm then decreased significantly (P=0.011) to $7.43\pm0.15\times10^5$ cfu/g in the day-15. In the control there was higher bacterial load ($2.87\pm0.10\times10^7$) cfu/g in the gill (Table 3). *Koi* intestine was more susceptible to bacterial infection and bacterial load was significantly decreased in skin, intestine and gill of *Koi* samples after oxytetracycline treatment (Figure 1).

Table 3: Changes in bacterial load of *Koi* skin, intestine and gill (cfu/g) at control and

Treatment	skin (Treated)	skin (Controlled)	intestine (Treat-)	intestine (Cont-)	gill (Treat-)	gill (Cont-)
Day-0	6.05×10^5	6.17×10^5	4.51×10^7	4.54×10^7	8.36×10^6	8.25×10^6
Day-1	7.21×10^5	6.48×10^5	5.8×10^7	4.66×10^7	8.83×10^6	8.53×10^6
Day-3	6.61×10^5	7.32×10^5	3.89×10^7	4.89×10^7	7.34×10^6	8.91×10^6
Day-5	3.01×10^5	7.89×10^5	1.65×10^7	5.32×10^7	5.92×10^6	9.52×10^6
Day-7	1.84×10^5	7.93×10^5	9.48×10^6	5.97×10^7	2.71×10^6	1.79×10^7
Day-9	8.38×10^4	8.41×10^5	8.13×10^6	6.38×10^7	9.48×10^5	1.95×10^7
Day-11	6.79×10^4	8.69×10^5	6.72×10^6	6.96×10^7	8.59×10^5	2.27×10^7
Day-13	5.35×10^4	8.98×10^5	4.37×10^6	7.73×10^7	7.18×10^5	2.64×10^7
Day-15	4.24×10^4	9.52×10^5	2.93×10^6	8.12×10^7	7.43×10^5	2.87×10^7

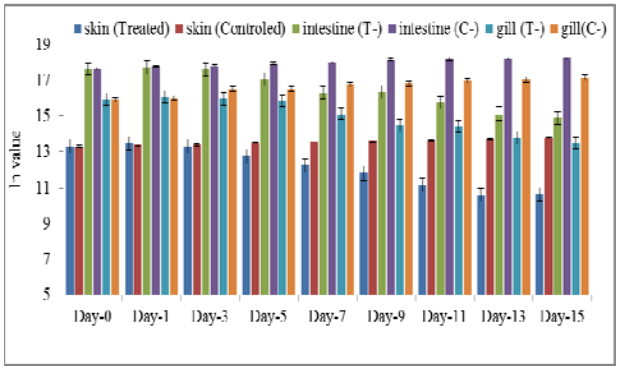

Fig 1: Changes in bacterial load of *Koi* skin, intestine and gill (cfu/g) in control and treated ponds.

Discussion

Gram-positive *Bacillus* spp. is generally more efficient in converting organic matter back to CO_2 than are gram negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime. Therefore the occurrence of *Bacillus* spp. in high numbers in the fish pond environments could be of immense benefit to the fish by changing the host-related or ambient microbial community (Verschuere *et al*. 2000) [22]. Gram-negative bacteria species dominated the isolates from the gill, *intestine* and *skin* of *Koi (Anabas testudineus). Flavobacterium* spp. is responsible for Bacteria Gill Disease (BGD) in salmonids which caused significant annual mortalities of salmonid in many European countries (Speare and Ferguson, 1989) [20]. Studies by Ampofo and Clerk (2010) [4] also reported the presence of

Flavobacterium spp. in cultured fish species. I*t is* alarming and causes infectious diseases both fish and consumers by this pathogenic bacterium *in Koi fish farm*. Zaman *et al*. (2014) [24] *found* five genera *like Pseudomonas* spp. (21.40%), *Aeromonas* spp. (33.46%), *Vibrio* spp. (14.78%), *Salmonella* spp. (21.40%) and *E. coli* (8.94%) *in Koi fish farm of Bangladesh* and were also confirmed by our results. *Shigella* spp. and *Pseudomonas* spp. can pose a threat to the health of the fish in the case of physiological and environment imbalance (Ampofo and Clerk, 2010) [4]. The presence of the coliform group of bacteria, mainly *Citrobacter*, *Enterobacter*, and *Escherichia* in fish and fish products presents a health hazard to humans (Fapohunda *et al*. 1994) [27]. Olayemi *et al*. (1991) [25] have reported that the presence of faecal coliform in fish intended for human consumption may constitute a potential danger not only in causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human infecting bacteria from non-aquatic sources. Some human pathogens such as *Aeromonas*, *Escherichia*, *Pseudomonas*, *Salmonella* and *Vibrio* have been found to survive and multiply in the gut, mucus and tissues of fish and thus render fish a potential vector of human disease over long periods (Allen *et al*. 1976) [26]. All these pathogens have been identified to be present in the tissues of fish by this study. *E. coli* in water or food is responsible for many gastro-intestinal diseases (Raina, 1999) [16]. *Escherichia coli* has been found in the intestinal tract of fish, gills, muscles and on the skin, when sewage water has been used to rear fish (Ampofo and Clerk, 2010) [4] also found in the present study. The most heavily contaminated parts are the intestines and the skin. Presence of *E. coli* in water or food indicates the possible presence of causative organisms of many gastro-intestinal diseases. Raj and Liston (1961) [17] found that some pathogenic and potentially pathogenic microorganisms including *E. coli*, *Staphylococcus* and some *anaerobes* survived when uncooked and precooked fish foods were stored at freezing temperatures. Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) was occurring for presence of *Vibrio* spp in shrimp ponds (Islam *et al*. 2017) [13]. *Vibrio cholerae* is responsible for the third-highest number of shellfish-related illnesses (Wittman and Flick, 1995) [23]. Detection of *Salmonella* spp. in seafood cannot be skipped as it is responsible for most of the food borne diseases or gastroenteritis characterized by diarrhea, abdominal cramp, vomiting, nausea, and fever. The isolation of bacteria species from the genus *Enterobacter*, *Streptococcus*, *Escherichia, Pseudomonas* and *Staphylococcus* from the fishes suggests that these potential human pathogens are present on fish farms. This means that contaminated fish from fish farms could pose serious health threat to humans when these bacteria are consumed in large quantities. *Shigella* spp. is also known to cause shigellosis in humans. Although the molecular basis of shigellosis is complex, the initial step in pathogenesis is penetration of the colonic mucosa. *Shigella* causes leakage of blood, inflammatory elements, and mucus into the intestinal lumen (Hale, 1991; Cabral, 2010) [8, 6]. This is an indication that all the organs are susceptible to bacterial infections. Now it has crying need to established fish farm biosecurity standard to verify *bacterial* contamination.

Conclusion

It is frightening that various infection causing pathogenic bacteria were found in *Koi* fish pond. In Bangladesh most of the fish consumed is bought directly from the fishermen and do not pass through any health safety checks. This can create instant serious health effect in the human body. So it would be ensured fish inspection program to regulate the quality of fresh fish from the farm or in the marketplace in Bangladesh. That may be a safeguard to protect the health of consumer.

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