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PATHOGENIC GUT MICROBIOTA ASSOCIATED WITH STRIPED CATFISH, *PANGASIANODON HYPOPHTHALMUS* CULTURED IN BANGLADESH AND THEIR ANTIBIOTIC SENSITIVITY PATTERN

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Abstract

To identify the gut bacteria of *Pangasianodon hypophthalmus* (striped catfish; Pangas) using classical and molecular approach was carried out. Total bacterial count (TBC) in the gut of pangas from farm and market samples were found 5.07 ± 1.70 × 10^6 and 1.40 ± 0.47 × 10^6 cfu/g, respectively. The gut microbiota of pangas was dominated by members of the Gram-negative genera. Only three isolates (MyF1/1, MyF1/4 and GaW1/2) were found to be Gram-positive among the 16 representative isolates. Using 16S rRNA gene sequencing; *Bacillus, Macrococcus, Citrobacter, Aeromonas, Proteus, Klebsiella, Enterobacter, Escherichia* and *Edwardsiella* were found to be associated with the gut of this fish. Among them, *Aeromonas* was the most dominant genus (5 out of 16). Antibiotic sensitivity pattern reflected that all the isolates were sensitive to gentamycin. Multiple antibiotics resistant isolates were also identified of which MyF3/13 (identified as *Citrobacter amalonaticus)* was found resistant against seven tested antibiotics. The presence of pathogenic bacteria in fish gut revealed the improper handling practices in fish market and unhygienic condition in the culture sites which might be a reason of fish-borne disease outbreaks. On the other hand, widespread use of various antibiotics in aquaculture without proper awareness may lead to resistance to multiple antibiotics.

Introduction

Pangasianodon hypophthalmus is popularly known as 'Pangas' (Spelled according to FishBase [http://fishbase.org/search.php]) in Bangladesh which belongs to the family Pangasiidae under the order Siluriformes. As the production volume of this fish species is high and is affordable to lower income consumers, it has become an essential fish for national food security in Bangladesh(1). The most advantageous aquaculture features of this fish are its rapid growth rate, large size, high market demand and 85% survival

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rate[2]. Farming of pangas is a significant component of aquaculture in Bangladesh with a total production of 494,357 tons in the fiscal year 2015 - 2016, accounting for 29% of the total farmed fish supply in the country[3]. Considering the great potentiality of pangas farming and its vital role in national food security, its production should be increased ensuring good quality. *Pangasius* catfish farming has been evolved to a shape of commercial enterprise over the last two decades in north-central part of Bangladesh, particularly in Mymensingh area [4]. Microorganisms present in fish gastrointestinal (GI) tracts are known to contribute to fish health. The gut is also a possible route for pathogenic microorganisms to invade and infect their host and finally limit the production of any commercially important fish species. A good number of studies suggested that gastrointestinal microbiota serve an important function in host metabolism, energy utilization and storage, immunity and health maintenance. The composition of gut microbiota can be affected by the host's genetic background, lifestyle and feeding behavior[5]. In freshwater fishes, *Aeromonas, Enterobacter, Pseudomonas, Micrococcus* and *Bacillus* are common in intestine. Human infections and intoxications with different bacteria transmitted from fish have been recorded *viz., Mycobacterium* spp., *Vibrio alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *V. cholerae*, *Escherichia coli*, *Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum*, *C. perfringens*, *Campylobacter jejuni*, *Edwardsiella tarda* etc.[6].

As fishes are significant to humans in terms of nutrition and economic value therefore, understanding of gut microbiota especially pathogenic gut microbiota is important for the purpose of improving fish health as well as aquaculture. Conventional culture methods may not accurately reflect the complete microbial composition in the gut of any fish species. Therefore, recent investigations with molecular approaches are now generating some accurate and exciting data. To minimize infections and contamination via microorganisms in fish production system, different antibiotics can be used. Proper selection of antibiotics is inevitable to suppress the activity of pathogenic microorganisms. Antibiotic susceptibility test or antibiogram of bacterial isolates is one way to observe susceptibility pattern of pathogenic microbes against different antibiotics. Therefore, the objective of this study was to identify pathogenic gut microbiota in *P. hypophthalmus* collected from different farms and markets along with antibiotic susceptibility of the representative bacterial isolates.

Materials and Methods

Eighteen Pangas fish samples (each site with three replicates) were collected from three Pangas farms of Mymensingh district (Muktagacha, Trishal and Valuka) and three wholesale markets in Sadar Upazilas of Gazipur, Mymensingh and Manikganj district. Then the gut samples from each fish were collected using a pair of sterile forceps and

scissors. Both the fish and gut samples were collected aseptically following the methods of American Public Health Association[7].

Collected gut samples were separately homogenized with physiological saline (PS) solution using sterilized homogenizer and serial dilution technique was employed using six different media i.e., nutrient agar (NA) (Oxoid LTD, Basingstoke, Hampshire, England) for total bacterial count (TBC), thiosulfate citrate bile salts sucrose (TCBS) (Oxoid Ltd., Basingstoke, Hampshire, England) for total *Vibrio* count (TVC), eosin methylene blue (EMB) (Himedia, India) for total coliform count (TCC), *Salmonella-Shigella* (SS) agar (Oxoid Ltd., Basingstoke, Hampshire, England) for total *Salmonella-Shigella* count (TSSC), mannitol salt agar (MSA) (Oxoid Ltd., Basingstoke, Hampshire, England) for total Staphylococcal count (TSC) and *Aeromonas* agar (AA) (Oxoid Ltd., Basingstoke, Hampshire, England) for total *Aeromonas* count (TAC). Inoculated duplicate plates were inverted and incubated at 37°C for 24 hrs in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach, Germany). After 24 hrs, plates having well discrete colonies were counted on a colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan). Isolates were selected primarily based on their different colony morphology and keeping representation of each colony type, some isolates were selected for detailed study. Selected isolates were purified through streak plate technique on respective agar media and were preserved as stock culture in Luria Bertany (LB) agar medium at 4°C and in LB broth medium with 30% glycerol at –80°C (cryopreservation).

Important physiological and biochemical tests *viz.,* Gram staining, motility test, starch hydrolysis test, Voges-Proskauer (V.P.) test, methyl red test, nitrate reduction test, production of indole, utilization of citrate, utilization of propionate, Kligler's Iron Agar (KIA) test, hydrolysis of gelatin, urease production test were carried out following standard manuals[8,9]. The bacteria were provisionally identified following Bergey's Manual of Systematic Bacteriology Vol. II[9] and Manual for Laboratory Investigations of acute enteric infections[10].

Molecular identification of the bacterial isolates was conducted by amplifying ~1500 bp fragments of 16S rRNA gene using 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-CGGTTACCTTGTTACGACTT-3′ primer pairs[11]. Amplification was conducted in a reaction volume of 25 µl containing 12.5 µl of hot start colorless master mix, 1 µl of DNA template, 9.5 µl of nuclease-free water, 1 µl of forward and reverse primer. PCR amplification was performed in an oil-free thermal cycler (Applied Biosystems 2720 Thermal Cycler) with following program: 95°C for 5 min for denaturation, then 32 cycles at 95°C for 30 sec, 48°C for 30 sec and 72°C for 1 min 30 sec, followed by an extension step at 72°C for 5 min. Successful amplification of the desired sequences were visualized by resolving the PCR products in 1% agarose gel (w/v) stained with 2 µl of ethidium bromide (H5041, Promega, USA). DNA bands were observed and photographed by AlphaImager MINI Gel documentation system (ProteinSimple, USA). Amplified DNA

were further purified with the Wizard PCR SV Gel and PCR Clean - Up System kit (Promega, USA) according to the manufacturer's instruction prior to sequencing. Sequencing of PCR products were performed using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to manufacturer's instruction and capillary electrophoresis was done using ABI Genetic Analyzer (Applied Biosystems, USA). To view DNA sequences, Geospiza's Finch TV version 1.4 was used. Sequences were analyzed through NCBI - BLAST database (http://blast.ncbi.nlm.nih.gov/) to find out possible similar organisms in the databases. MEGA v 7.0 was used for constructing phylogenetic tree for finding the taxonomic positions of the isolates.

The Kirby-Bauer disc diffusion method[12] on Muller Hinton media was used to determine the antibiotic sensitivity or resistance pattern of the selected bacterial isolates for 12 antibiotics *viz.,* amoxycillin (10 μg), ampicillin (10 μg), azithromycin (15 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), nitrofurantoin (300 μg), polymyxin B (300 unit), streptomycin (10 μg), sulphamethoxazole (25 μg) and tetracycline (30 μg).

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v. 20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). One way ANOVA was performed to test the significance at 5% level.

The reported sequences in this study have been submitted to GenBank database under accession numbers MH220299 to MH220313.

Results and Discussion

Quantitative enumeration of gut microbiota: Comparative analysis of bacterial load in gut of pangas fish among different farms and markets showed some variations (Table 1). No significant difference was observed among 18 gut samples of pangas collected from three farms and three markets in case of TBC, TVC, TSSC, TAC, TSC and TCC ($p > 0.05$). The highest TBC was found in Trishal farm ($5.92 \pm 5.04 \times 10^6$ cfu/g) among three farms; and among the wholesale markets, highest count was observed in Gazipur market ($1.70 \pm 0.08 \times 10^6$ cfu/g). It is considered that the fish gut harbors about 10^7 - 10^8 cfu/g bacteria[13] which supports the present findings. Total *Staphylococcal* count ranged from $4.63 \pm 3.18 \times 10^4$ cfu/g (Valuka farm) to $1.97 \pm 1.72 \times 10^5$ cfu/g (Muktagacha farm). On the other hand, highest Staphylococcal count was found in Gazipur market ($1.61 \pm 0.8 \times 10^5$) cfu/g. In a previous study, total Staphylococcal count in the gut of another fish *Clarias gariepinus* was recorded to be $3.58 \pm 0.04 \times 10^5$ cfu/g[13]. Enteric and related bacterial count on EMB agar plate were ranged from $5.5 \pm 1.61 \times 10^4$ (Valuka) to $2.29 \pm 2.10 \times 10^5$ (Muktagacha) in farm samples. In wholesale market samples, total coliform count ranged from $1.52 \pm 0.7 \times 10^4$ (Mymensingh) to $1.62 \pm 1.09 \times 10^5$ cfu/g (Gazipur). Among the fish samples of farm, the highest total *Aeromonas* count was observed in the gut samples of Pangas collected from Muktagacha farm ($2.42 \pm 0.82 \times 10^5$ cfu/g) and among the fish samples of markets,

the highest total *Aeromonas* count was observed in the gut samples of pangas collected from Gazipur wholesale market (7.38 ± 7.31 × 10^4 cfu/g). Total *Salmonella* and *Shigella* count ranged from 7.85 ± 4.7 × 10^4 (Valuka) to 3.52 ± 1.32 × 10^5 cfu/g (Muktagacha) in farm samples. On the other hand, among wholesale markets, highest *Salmonella-Shigella* count (6.56 ± 6.22 × 10^4 cfu/g) was found in Gazipur market. Highest *Vibrio* count was found in Muktagacha farm (2.72 ± 1.24 × 10^4 cfu/g) and in Manikganj wholesale market (3.09 ± 1.03 × 10^4 cfu/g). According to International Commission on the Microbiological Specification of Foods [14] guideline, acceptable limit of total bacterial counts, total *Staphylococcal* count, total coliform count, total *E. coli* count, total *Salmonella* count, total *Shigella* count and total *Vibrio cholerae* counts for white fish are 5 × 10^5, >10^3, 100, 0, 0, 1.0 × 10^2 and 0 cfu/g, respectively. Present findings of the gut bacterial density of pangas fish of both farm and markets exceed these limits. So, this study clarified that the collected pangas samples are not microbiologically safe.

Table 1. Gut bacterial density (cfu/g; mean ± SEM) of 18 pangas fish from three farms and three wholesale markets measured on NA, MSA, EMB, *Aeromonas*, SS and TCBS agar media

Bacterial density	Bacterial density (cfu/g) (Mean ± SEM)					
	Farm			Wholesale market		
	Muktagacha	Trishal	Valuka	Gazipur	Mymensingh	Manikganj
TBC	5.9 ± 2.56 × 10^6	5.92 ± 5.04 × 10^6	3.4 ± 0.7 × 10^6	1.70 ± 0.84 × 10^6	8.48 ± 3.77 × 10^5	1.66 ± 1.25 × 10^6
TSC	1.97 ± 1.71× 10^5	5.20 ± 4.4 × 10^4	9.8 ± 5.75 × 10^4	1.61 ± 0.84 × 10^5	3.68 ± 1.67 × 10^4	4.87 ± 1.87 × 10^4
TCC	2.29 ± 2.10 × 10^5	1.40 ± 0.64 × 10^5	5.50 ± 1.61 × 10^4	1.62 ± 1.09 × 10^5	1.52 ± 0.74 × 10^4	2.51 ± 1.30 × 10^4
TAC	2.42 ± 0.82 × 10^5	9.70 ± 6.60 × 10^4	3.43 ± 1.42 × 10^4	7.38 ± 7.31 × 10^4	6.23 ± 1.39 × 10^4	5.84 ± 4.71 × 10^4
TSSC	3.52 ± 1.32 × 10^5	2.35 ± 1.83 × 10^5	2.22 ± 0.78 × 10^5	6.56 ± 6.22 × 10^4	4.96 ± 2.05 × 10^4	1.91 ± 1.31 × 10^4
TVC	2.72 ± 1.24 × 10^4	1.09 ± 0.06 × 10^3	1.84 ± 1.34 × 10^4	1.96 ± 1.82 × 10^4	1.53 ± 0.67 × 10^4	3.09 ± 1.03 × 10^4

Provisional Identification of the bacterial isolates: During this study, a total of 200 colonies (100 from farm and 100 from market samples) were primarily selected from different selective agar medium based on different colony morphology. Among them, 36 isolates representing those 200 colonies were selected and purified equally from farm and markets based on their characteristics, growth condition and suspected diversity. Then 16 robustly grown isolates were finally selected and purified for further study towards identification keeping in mind that the representative isolates were picked from the highest possible diversified samples. Table 2 illustrates the provisionally identified bacterial isolates through biochemical tests. Of the 16 representative isolates, 3 were Gram-positive and the remaining 13 were Gram-negative bacteria. All isolates were found to be catalase, nitrate and gelatin positive. All Gram-negative isolates could produce urease except one (MyF3/13). Among the 13 Gram-negative isolates, 9 could ferment glucose only, one could ferment both glucose and lactose (MyW2/8) and another 3 were non fermenter (MyF2/7, MyW2/10, MaW2/13). All the isolates produced gas

except 2 (MyF2/7, MyF3/15). Among the isolates, only one formed H_2S (MaW2/13). *Bacillus* and *Staphylococcus* were found as Gram-positive genera. The 13 Gram-negative isolates were identified as *Aeromonas* sp. (6), *Klebsiella* sp. (2), *Enterobacter* sp. (1), *Proteus rettgeri* (2), *P. mirabilis* (1) and *P. morganii* (1). *Aeromonas hydrophila* is the most common and frequently occurring bacteria found in freshwater habitats throughout the world causing diseases among cultured and wild fishes[15].

Molecular identification of bacterial isolates: The gel photographs (Fig. 1A,B) show the size of the PCR product (1.5 kb) of 16S rDNA of 16 bacterial isolates. Identification through 16S rRNA gene sequencing of 15 (one omitted because of noisy chromatogram) representative isolates through nucleotide BLAST of NCBI and their comparison to provisional identification is summarized in Table 3. Among 15, nine isolates (60%) matched according to their provisional identification in generic level.

Five isolates were identified as *Aeromonas* sp. whereas two (MyF1/1 and GaW1/2) were identified as *Bacillus aryabhattai* and *B. tequilensis* among 15 isolates in molecular identification. Another two MyF1/6 and MyF3/13 were identified as *Citrobacter freundii* and *C. amalonaticus*. Other isolates like MyF1/4, GaW1/1, GaW3/5, MyW2/10, MaW2/13 and MaW1/14 were identified as *Macrococcus caseolyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter ludwigii*, *Escherichia coli* and *Edwardsiella tarda*, respectively. *Aeromonas* is regarded as an important disease-causing pathogen of fish as well as in human and *A. salmonicida* included as a predominant species in fish and water samples[16]. *Bacillus, Proteus, Pseudomonas, Klebsiella, Streptococcus, Salmonella, Staphylococcus, Micrococcus, Serratia* and *Escherichia* are also found in the intestine of fish[17]. Effective control of microflora in fish intestines is possible using antibiotic-producing bacteria. A similar approach may be possible in freshwater fish using intestinal bacteria with an inhibitory effect against pathogenic bacteria[16]. Several solutions can also be suggested, that aim at the development of sustainable aquaculture practices, such as those including the use of probiotics, essential oils to increase immune status of fish[18] as well as the adoption of measures able to warrant the fast abatement of antimicrobial residues in animal wastes[19]. Another possible solution to chemotherapies in aquaculture is related to the use of vegetable extracts[20]. Nevertheless, further studies are needed to assess any potential impact of these substances on the host microbiota and on the environment.

Phylogenetic analysis: Phylogenetic tree (Fig. 2) was constructed based on the partial 16S rRNA gene sequences of the 15 representative isolates and 21 downloaded sequences (NCBI GenBank Nucleotide database) using neighbor-joining and BioNJ algorithms which confirmed the taxonomic position of the isolates. It is clear from the phylogenetic tree that MyF1/1 and GaW1/2 were closely related which supported their similarity with *Bacillus* sp. The strain MyF1/4 showed similarity with *Macrococcus caseolyticus* NW_A28_MG543841. The phylogenetic tree also confirms the taxonomic position of

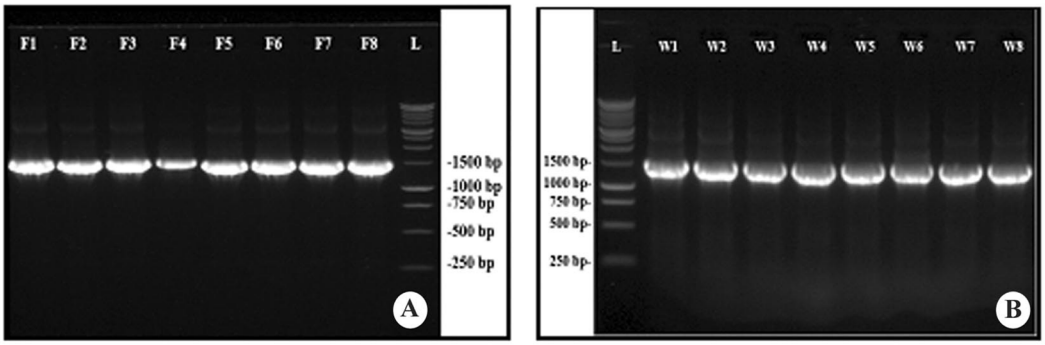

Fig. 1. PCR product of 16S rDNA generated from 16 bacterial isolates. Isolates of Farm: F1, F2, F3, F4, F5, F6, F7, F8 (A) and isolates of wholesale market: W1, W2, W3, W4, W5, W6, W7, W8 (B) whereas L denotes DNA ladder, 1 kb (Marker)

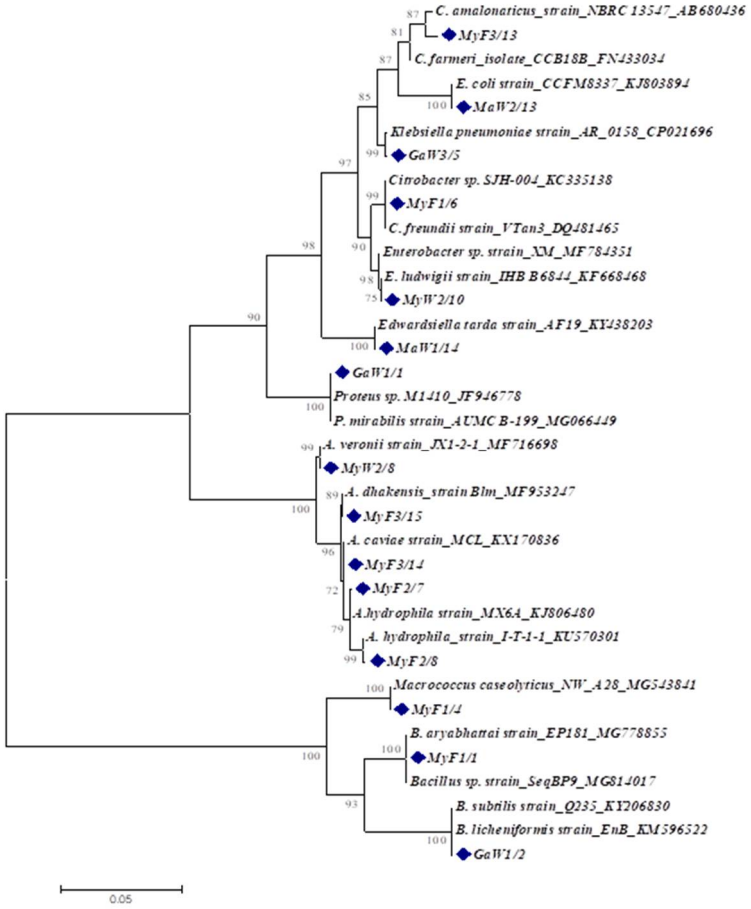

Fig. 2. The neighbor-joining (NJ) phylogenetic tree of the representative 15 bacterial isolates from present study based on partial 16S rRNA gene sequences constructed by MEGA v. 7. Numbers in trees are bootstrap values. Blue diamond shape indicates position of the studied strains. There were 36 nucleotide sequences each with 1326 bp.

Table 2. Physiological and biochemical characteristics of the representative 16 isolates from farm and marketed pangas fish.

Bacterial isolates	Oxidase test	Motility test	Starch hydrolysis test	V.P. test	M.R. test	Citrate test	Propionate test	Indole test	Urease test	Kligler's iron agar test				Provisional identification
										Butt	Slant	Gas	HS	
MyF1/1	−	+	+	+	+	+	+	−	ND	ND	ND	ND	ND	*Bacillus brevis*
MyF1/4	−	+	−	−	+	+	−	−	ND	ND	ND	ND	ND	*Staphylococcus gallinarum*
MyF1/6	−	+	+	+	+	+	+	+	+	A	K	+	−	*Proteus rettgeri*
MyF2/7	+	+	+	−	+	+	−	+	+	K	K	−	−	*Aeromonas* sp.
MyF2/8	+	+	+	−	+	−	−	+	+	A	K	+	−	″
MyF3/13	+	+	+	−	+	+	−	+	−	A	K	+	−	″
MyF3/14	+	+	+	−	+	+	−	−	+	A	K	+	−	″
MyF3/15	+	−	+	+	+	+	+	+	+	A	K	−	−	″
GaW1/1	−	−	−	+	+	+	+	−	+	A	K	+	−	*Proteus morganii*
GaW1/2	−	+	+	+	+	+	+	−	ND	ND	ND	ND	ND	*Bacillus subtilis*
GaW3/5	−	−	+	+	−	+	+	−	+	A	K	+	−	*Klebsiella* sp.
MyW2/8	−	−	−	−	+	+	+	−	+	A	A	+	−	″
MyW2/10	−	+	+	+	+	+	+	−	+	K	K	+	−	*Enterobacter* sp.
MaW1/11	−	+	+	+	+	+	+	+	+	A	K	+	−	*Aeromonas* sp.
MaW2/13	−	+	−	+	+	+	+	+	+	K	K	+	+	*Proteus mirabilis*
MaW1/14	−	+	−	+	+	+	+	+	+	A	K	+	−	*Proteus rettgeri*

'+' indicates oxidase, V. P., M. R. test positive; Indole and urease produced; Starch hydrolyzed; Citrate, propionate utilized and motile.
'-' indicates oxidase, V. P., M. R. test negative; Indole and urease not produced and starch not hydrolyzed; Citrate, propionate unutilized and non-motile.
ND = Test not done, A = Acidic, K = Alkaline.

Table 3. 16S rRNA sequence based identification of 15 representative bacterial isolates from Farm and Market samples of pangas.

Isolates ID	Provisional identification	Molecular identification	Strain	No. of base pairs used for molecular identification	Total score	Query cover (%)	E-value	Identity match (%)	GenBank Acc. No. of corresponding sequence	GenBank Acc. No. of isolates under present study
MyF1/1	*Bacillus brevis*	*Bacillus aryabhattai*	A36	1417	2617	100	0.0	100	KU323599	MH220299
MyF1/4	*Staphylococcus gallinarum*	*Macrococcus caseolyticus*	GCF1S3	1402	2590	100	0.0	100	MG744632	MH220300
MyF1/6	*Proteus rettgeri*	*Citrobacter freundii*	AR_0023	1387	20299	100	0.0	99	CP026677	MH220301
MyF2/7	*Aeromonas* sp.	*Aeromonas hydrophila*	M-X6A	1390	2556	99	0.0	99	KJ806480	MH220302
MyF2/8	"	"	I-N-1-1	1390	2551	100	0.0	99	KU570297	MH220303
MyF3/13	"	*Citrobacter amalonaticus*	NBRC 13547	1376	2475	100	0.0	99	AB680436	MH220304
MyF3/14	"	*Aeromonas caviae*	MCL	1407	2599	100	0.0	100	KX170836	MH220305
MyF3/15	"	*A. dhakensis*	Blm	1394	2575	100	0.0	100	MF953247	MH220306
GaW1/1	*Proteus morganii*	*Proteus mirabilis*	FDAARGOS	1407	18093	100	0.0	99	CP026051	MH220307
GaW1/2	*Bacillus subtilis*	*Bacillus tequilensis*	FJAT-40023	1395	2577	100	0.0	100	MG905895	MH220308
GaW3/5	*Klebsiella* sp.	*Klebsiella pneumoniae*	AR_0158	1394	20314	100	0.0	99	CP021696	MH220309
MyW2/8	"	*Aeromonas veronii*	FZ1SY	1404	2593	100	0.0	100	MF716714	MH220310
MyW2/10	*Enterobacter* sp.	*Enterobacter ludwigii*	IHB B 6844	1401	2588	100	0.0	100	KF668468	MH220311
MaW1/11	*Aeromonas* sp.	Omitted from the analysis due to noisy chromatogram of the sequence								
MaW2/13	*Proteus mirabilis*	*Escherichia coli*	FORC	1382	17767	100	0.0	100	CP025318	MH220312
MaW1/14	*P. rettgeri*	*Edwardsiella tarda*	AF19	1407	2599	100	0.0	100	KY438203	MH220313

Table 4. Antibiogram for *P. hypophthalmus* gut bacterial isolates against 12 antibiotics.

Antibiotics	Isolates of farm samples (N = 8)			Isolates of market samples (N = 8)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Amoxicillin (AML)	4 (50)	2 (25)	2 (25)	3 (37.5)	3 (37.5)	2 (25)
Ampicillin (AMP)	5 (62.5)	2 (25)	1 (12.5)	3 (37.5)	3 (37.5)	2 (25)
Azithromycin (AZM)	0 (0)	1 (12.5)	7 (87.5)	1 (12.5)	0 (0)	7 (87.5)
Chloramphenicol (C)	0 (0)	0 (0)	8 (100)	5 (62.5)	2 (25)	1 (12.5)
Erythromycin (E)	5 (62.5)	2 (25)	1 (12.5)	0 (0)	6 (75)	2 (25)
Gentamicin (CN)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)
Kanamycin (K)	1 (12.5)	0 (0)	7 (87.5)	0 (0)	4 (50)	4 (50)
Nitrofurantoin (F)	1 (12.5)	0 (0)	7 (87.5)	1 (12.5)	2 (25)	5 (62.5)
Polymyxin B (PB)	0 (0)	0 (0)	8 (100)	6 (75)	0 (0)	2 (25)
Streptomycin (S)	0 (0)	5 (62.5)	3 (37.5)	1 (12.5)	0 (0)	7 (87.5)
Sulphamethoxazole (SXT)	1 (12.5)	1 (12.5)	6 (75)	0 (0)	2 (25)	6 (75)
Tetracycline (TE)	1 (12.5)	0 (0)	7 (87.5)	1 (12.5)	1 (12.5)	6 (75)

*S = Sensitive, I = Intermediate, R = Resistant, N = Number of Isolates.

Table 5. Susceptibility of 16 representative bacterial isolates from gut samples of Pangas against 12 tested antibiotics.

Isolates name	Antibiotics		
	Sensitive	Intermediate	Resistant
MyF1/1	AML, F, S, CN, SXT, PB, K, C, TE, AZM	E	AMP
MyF1/4	AML, F, S, E, CN, SXT, PB, AMP, K, C, TE, AZM		
MyF1/6	F, CN, SXT, PB, K, C, TE, AZM	AML, S, AMP	E
MyF2/7	F, CN, SXT, PB, K, C, TE, AZM	S	AML, E, AMP
MyF2/8	F, S, CN, SXT, PB, K, C, TE, AZM	E	AML, AMP
MyF3/13	CN, PB, C, AZM	S	AML, F, E, SXT, AMP, K, TE
MyF3/14	F, CN, SXT, PB, K, C, TE	AML, S, AMP, AZM	E
MyF3/15	F, CN, PB, K, C, TE, AZM	S, SXT	AML, E, AMP
GaW1/1	E, CN, SXT, PB, K, AZM	AMP, C	AML, F, S, TE
GaW1/2	F, S, CN, SXT, K, AZM	E, TE	AML, PB, AMP, C
GaW3/5	AML, F, S, CN, AMP, TE, AZM	E, SXT, K	PB, C
MyW2/8	S, E, CN, SXT, PB, AMP, TE, AZM	AML, F, K	C
MyW2/10	F, S, CN, SXT, K, TE, AZM	AML, E	PB, AMP, C
MaW1/11	F, S, CN, K, TE, AZM	E, SXT, C	AML, PB, AMP
MaW2/13	AML, S, CN, SXT, C, TE, AZM	F, E, AMP, K	PB
MaW1/14	F, S, CN, SXT, TE	AML, E, AMP, K	PB, C, AZM

MyF2/7, MyF2/8, MyF3/14, MyF3/15 and MyW2/8 under the genus *Aeromonas*. The tree also supports the taxonomic position of MyF3/13, MaW2/13, GaW3/5, MyF1/6, MyW2/10, MaW1/14 and GaW1/1 as the genus *Citrobacter, Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Edwardsiella* and *Proteus*, respectively.

Antibiotic susceptibility of the representative bacterial isolates: Sixteen bacterial isolates were tested against 12 common antibiotics and results are shown in Tables 4 and 5. All farm isolates showed 100% sensitivity to gentamycin, polymyxin B and chloramphenicol whereas market isolates only to gentamicin. The farm isolates showed the lowest level of sensitivity (12.5%) against erythromycin and ampicillin; and market isolates only to chloramphenicol. No farm isolate showed resistance to streptomycin, gentamicin, polymyxin B, chloramphenicol and azithromycin. On the other hand, no market isolate showed resistance to erythromycin, gentamicin, sulphamethoxazole and kanamycin. Highest resistance level (62.5%) was found against erythromycin and ampicillin among farm isolates. On the other hand, market isolates showed highest resistance level (75%) against polymyxin B. The high resistance emerges because of treating infections or diseases with different antibacterial drugs[21] and these are limiting the value of the antibiotics in the control of bacterial diseases of fish[22]. Multiple drug resistance has been reported in a good number of studies of fish pathogens and aquaculture environments[23]. Different multiple drug resistant bacteria were found in this study (Table 5) *viz.,* isolate MyF3/13 (resistant against 7 antibiotics), GaW1/1 (resistant against 4 antibiotics), GaW1/2 (resistant against 4 antibiotics) etc. Only one farm isolate MyF1/4 showed sensitivity to all antibiotics. Widespread use of antibiotics in the aquaculture systems in Bangladesh may act as the source of antibiotics diffusion[24] that may exert selective pressure on bacterial flora to be resistant. Therefore, the frequent use of antibacterial drugs in aquaculture farms may be the reason of the resistance of bacteria to different antibiotics. There is an urgent need to strengthen and improve the capacity of existing government institutions to provide advice on disease prevention and control, e.g. establishing effective national disease diagnostic surveillance systems, and access to quality products, but also to identify new ways, e.g. involving both public and private partners[25]. Also, certification schemes to promote the prudent use of antimicrobials and other compounds should be considered. Probiotics may offer alternatives to antimicrobial compounds, which may reduce the risk of antibacterial resistance[26].

From the above results it can be confirmed the presence of pathogenic bacteria in the gut of Pangas, which are of public health concern. Therefore, a careful handling is required to prevent cross contamination from gut to other parts during processing and preservation. This study also confirms the existence of multiple antibiotic resistant bacteria in the gut of Pangas, which may be the outcome of indiscriminate use of antibiotics in culture area. Use of probiotics as an alternative to the antimicrobial compounds or vaccines may be an effective solution to this problem[27].

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References

1. Ali H, MM Haque and B Belton 2013. Striped catfish (*Pangasianodon hypophthalmus,* Sauvage, 1878) aquaculture in Bangladesh: An overview. Aquac. Res. **44**(6): 950-965.

2. Rahman MK, JN Akhter, MA Mazid and GC Haldar 1992. Comparison of fingerling growth rates and survival of two *Pangasius* species. J. Inland Fish. Soc. India. **24**(2): 40-44.

3. DoF 2017. *Yearbook of Fisheries Statistics of Bangladesh 2016-17.* Fisheries Resources Survey System (FRSS), Department of Fisheries, Bangladesh: Director General, DoF, 2017. **34**: 129.

4. Ali H and MM Haque 2011. Impacts of *Pangasius* aquaculture on land use patterns in Mymensingh district of Bangladesh. J. Bangladesh Agri. Uni. **9**(1): 169-178.

5. Yan Q, CJ Van Der Gast and Y Yu 2012. Bacterial community assembly and turnover within the intestines of developing zebrafish. Plos One **7**(1): e30603.

6. Novotny L, L Dvorska, A Lorencova, V Beran and I Pavlik 2004. Fish: A potential source of bacterial pathogens for human beings. Vet. Med. Czech. **49**(9): 343-358.

7. APHA (American Public Health Association) 1998. *Standard Methods for the Examination of Water and Wastewater.* 20th ed. APHA, Washington DC, USA, pp. 1325.

8. SAB (Society of American Bacteriologists) 1957. *Manual of Microbiological Methods.* McGraw Hill Book Company Inc, New York, pp. 315.

9. Sneath PHA, MES Mair, JG Holt 1986. *Bergey's Manual of Systematic Bacteriology.* 9th ed. Williams and Wilkins Company, Baltimore, London, pp. 1122-1129.

10. WHO 1987. *Manual for Laboratory Investigations of Acute Enteric Infections*. CDD/83.3 Rev. 1, WHO (World Health Organization), Geneva, pp. 17.

11. Weisburg WG, SM Barns, DA Pelletier and DJ Lane 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173**(2): 697-703.

12. Hudzicki J 2009. *Kirby-Bauer Disc Diffusion Susceptibility Test Protocol.* American Society for Microbiology, Washington, DC, pp. 23.

13. Perez T, JL Balcazar, I Ruiz-Zarzuela, N Halaihel, D Vendrell and ID Blas 2010. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal. Immunol. **3**: 355-360.

14. ICMSF (International Commission on Microbiological Specifications for Foods) 1986. *Sampling plans for fish and shellfish.* 2nd ed. Microorganisms in Foods. Sampling for Microbiological Analysis: Principles and Scientific Applications. ICMSF, Vol. 2, University of Toronto Press, Toronoto. (https://seafood.oregonstate.edu/sites/agscid7/files/snic/sampling-for-microbiological-analysis-principles-and-specific-applications-icmsf.pdf; accessed on 14 September, 2018)

15. Cipriano RC 2001. *Revision of fish disease leaflet 68 (1984), Aeromonas hydrophila and Motile Aeromonad Septicemias of Fish*, by Cipriano RC, Bullock GL and Pyle SW. (https://articles.extension.org/sites/default/files/w/1/1e/Aeromonas_hydrophila.pdf; accessed on 14 September, 2018)

16. Janda JM and SL Abbott 2010. The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. Clin. Microbiol. Rev. **23**(1): 35-73.

17. Tanu DDD, R Khandeparker, RA Sreepada, SV Sanaye and HB Pawar 2012. A study on bacteria associated with the intestinal tract of farmed yellow seahorse, *Hippocampus kuda* (Bleeker, 1852): Characterization and extracellular enzymes. Aquac. Res. **43**: 386-394.

18. Romero J, CG Feijoo and P Navarrete 2012. Antibiotics in aquaculture-use, abuse and alternatives. *In:* Carvalho ED, David GS, Silva RJ (Eds.), Health and Environment in Aquaculturem In Tech 6.

19. The Review on Antimicrobial resistance 2015. Antimicrobials in agriculture and the environment: reducing unnecessary use and waste, O'Neill J, 1-44.

20. Caruso G 2016. Antibiotic resistance in fish farming environments: A global concern. Journal of Fisheries Sciences **10**(4): 9.

21. Sorum H 1999. Antibiotic resistance in aquaculture. Acta. Vet. Scand. Suppl. **92**: 29-36.

22. Smith P, M Hiney and O Samuelson 1994. Bacterial resistance to antimicrobial agents used in fish farming: A critical evaluation of method and meaning. Annu. Rev. Fish Dis. **4**: 273–313.

23. Hatha M, AA Vivekanandam, GJ Joice and GJ Christol 2005. Antibiotic resistance pattern of motile aeromonads from farm raised freshwater fish. Int. J. Food Microbiol. **98**: 131-134.

24. Sorum H 2006. *Antimicrobial Resistance in Bacteria of Animal Origin. In:* Antimicrobial Drug Resistance in Fish Pathogens. American Society for Microbiology Press, Washington DC, USA, pp. 213-238.

25. Kim CTT, H Jesper, P Clausen, V Thi, B Tersbøl and A Dalsgaard 2017. Use practices of antimicrobials and other compounds by shrimp and fish farmers in Northern Vietnam. Aquaculture Reports **7**: 40-47.

26. Watts J, H Schreier, L Lanska and M Hale 2017. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Marine Drugs **15**(6): 158.

27. Cruz PM, AL Ibanez, OAM Hermosillo and HC Ramirez Saad 2012. Use of probiotics in aquaculture. ISRN Microbiology. Article ID 916845, pp. 13.

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