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GUT BACTERIA OF SNAKEHEADS OF CHINADI BEEL, NARSINGDI AND THEIR ANTIBIOGRAM PROFILE

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Abstract

The gut bacteria of snakehead fish (*Channa punctata*, *C. marulius* and *C. striata*) collected from Chinadi beel, Narsingdi, Bangladesh was characterized and formulated their antibiogram profile. The mean richness of intestinal bacteria in *C. punctata* was significantly higher in comparison to *C. marulius* and *C. striata*. A total of 24 isolates under the genus *Bacillus*, *Enterobacter*, *Citrobacter*, *Kluyvera*, *Vibrio*, *Pseudomonas* and *Acinetobacter* were identified. The strains of *Enterobacter* and *Pseudomonas* showed 100% resistance to eight antibiotics. The isolates of *C. punctata* were found to be resistant to nine antibiotics followed by *C. marulius* (8) and *C. striata* (7) indicating a critical condition of Chinadi beel of being polluted by anthropogenic activities.

Introduction

Chinadi beel is prominent in the district, Narsingdi comprising of 340 acres connected to Shitalakshya river. This beel has a diversified fish population of 51 species under 7 orders and 19 families. *Channa punctata* and *C. striata*, commonly known as spotted snakehead and striped snakehead, are categorized as "least concern" in the IUCN conservation fish status for Chinadi beel whereas *C. marulius*, or the great snakehead, is listed as “endangered”. Since people of surrounding villages usually consume fish captured from this waterbody, the overall health of the beel plays a key role in public health. However, the condition of the waterbody as well as its associated organisms are gradually deteriorating due to natural and anthropogenic activities (Majumdar *et al*. 2020).

The fish community offers the most favorable growth environment to the microbes inside their gut due to being rich in nutrients (Banerjee and Ray 2017, Kim *et al*. 2021). The abundance and diversity of these gut microbes greatly vary depending on the developmental stage, gut structure, diet composition, trophic level, habitat, and surrounding environment (Nayak 2010, Sullum *et al*. 2012). As the contaminated waterbody contains a densely populated bacterial community, fish from that region harbor more microbes as well. Thus, in sanitary biology, certain microbes of the gut of aquatic organism act as a biological indicator of the water condition of a specific place.

Since no records have been found regarding the water condition of Chinadi beel, the aim of this study was to understand the water quality by enumerating and comparing the gut microbial communities in the snakeheads that cohabited in Chinadi beel by identifying microbes based on their physiological and molecular characteristics, as well as by assessing their response to different antibiotics.

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Materials and Methods

Random sampling of apparently healthy *Channa punctata*, *C. marulius* and *C. striata* were done using cast net and fish trap from Chinadi beel located at Shibpur, Narsingdi. The geographical location of the study area is comprised of 24°03'38.5"N and 90°40'25.5"E. The investigation was carried out from December 2020 to May 2021 and nine samples were studied. After capturing, the live fishes were transported to the laboratory. The average weight, total length and gut length were measured. The gut was minced and homogenized with 0.89% physiological saline in a 1:10 ratio (Das and Tripathi 1991). Constantly diluting up to 10^4 dilutions, 1 ml of sample was plated by pour plate technique onto sterilized Peptone Yeast Extract Glucose (PYG) agar, Eosin Methylene Blue (EMB) agar, Cetrimide agar and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar. The samples were spread thoroughly, and the plates incubated at 37°C for 24 hrs.

The preponderant group of bacterial colonies from the media were chosen based on their morphological and microscopic characterization. The isolates were subjected to physiological characterization (Table 1). The growth response of the isolates was recorded at different temperatures and pH (Garrity 2005, Whitman 2009).

For molecular characterization of the 16S rRNA gene of the selected isolates, PCR based amplification technique was followed by using universal primers as CC [F] 5'-CAGAC TCCTACGGGAGGCAGCC-3′ and CD [R] 5′-CTTGTGCGGGCCCCCGTCAATTC-3'. The heat lysis method was used for DNA extraction from bacterial isolates (Rahman *et al*. 2014). For amplification of 16S rDNA, 12.5 µl of master mix (PROMEGA, Madison, USA) was used for each isolate. The amplified products were checked for desired amplicon in 1% agarose gel. The amplified DNA was further purified with the Monarch PCR and DNA Cleanup kit (New England Biolabs Inc., MA, USA). The sequences were analyzed through BLAST for comparing primary sequence information.

To understand the intergeneric relationship of the bacterial isolates, a neighbor-joining phylogenetic tree was constructed using MEGA X software (Kumar *et al*. 2018). The reference sequences were submitted for accession number to the GenBank/NCBI database. MUSCLE was used to perform nucleotide multiple sequence alignments. Following the Kimura two-parameter (K2P) model, the evolutionary distances between these representative isolates were calculated using the neighbor-joining distance-based algorithm with a bootstrapping value of 1,000 replications. (Kimura 1980, Felsenstein 1985, Saitou and Nei 1987).

The Kirby-Bauer disc diffusion method was performed to understand the susceptibility of the isolates against 11 antibacterial compounds. Selected bacterial isolates were inoculated in nutrient broth and incubated for 24 hrs and then the broth culture was spread on the surface of Mueller–Hinton Agar (MHA). The antibiotic discs were placed on the surface of the agar plates and incubated at 37°C for 24 hrs. Finally, the zone of inhibition was measured to detect susceptibility of the bacteria following the guidelines of Hudzicki (2009).

Results and Discussion

The present study revealed a significant difference in bacterial counts among the agar media used in the experiment (Fig. 1). Highest bacterial counts were observed on PYG agar medium and average heterotrophic bacterial count was maximum (2.63×10^5 cfu/g) in the samples collected from the gut of *C. punctata*. Since *C. punctata* resides in shallow water consuming debris or other microbe-contaminated foods whereas the other two species are the inhabitants of deeper water, consuming larger aquatic organisms. Furthermore, *C. punctata* attains a smaller body size in proportion to *C. marulius* and *C. striata*, indicating the increased chances of susceptibility to

pathogens which supports the study findings. The average enteric bacterial load in *C. punctata* was measured to be 7.7×10^3 cfu/g on EMB plates which was almost seven times higher than that of *C. marulius* and *C. striata*. The mean *Vibrio* count on TCBS agar plates was higher in *C. punctata* as well measuring 7.84×10^4 cfu/g indicating the higher possibility of contamination in the sampling site with sewage disposal. Compared to rest of the culture media, few bacterial colonies were observed in cetrimide media in case of *C. striata*. However, *C. marulius* samples showed no growth and *C. striata* samples showed maximum *Pseudomonas* counts of 2×10^2 cfu/g.

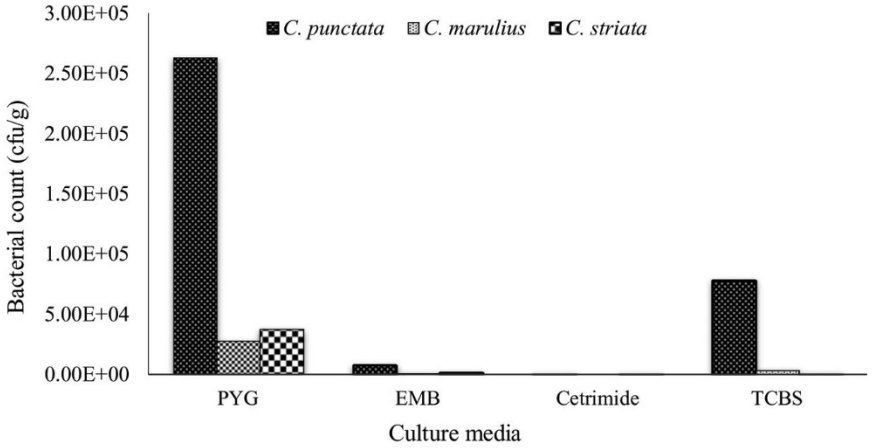

Fig. 1. Bacterial counts on PYG, EMB, Cetrimide and TCBS agar media.

After initial screening of 94 bacterial isolates, a total of 24 isolates were selected for the study. The bacterial species were *Bacillus* spp. (7), *Enterobacter* spp. (6), *Citrobacter* spp. (5), *Kluyvera cryocresens* (2), *Pseudomonas aeruginosa* (2), *Vibrio parahaemolyticus* (1) and *Acinetobacter* sp. (1) (Tables 1 and 2). The isolates were comprised of Gram-positive *Bacillus* (29%) and Gram-negative *Enterobacter* (25%), *Citrobacter* (21%), *Kluyvera* (9%), *Pseudomonas* (8%), *Vibrio* (4%), and *Acinetobacter* (4%).

16S rDNA gene amplification of seven isolates was confirmed by agarose gel electrophoresis (Fig. 2). The selected gene amplified isolates were identified up to species epithet (Table 3). Isolate CS-P-21 was identified as *Acinetobacter baumanii* which is an opportunistic pathogen in fish and previously recorded in *Channa striata* (Rauta *et al.* 2011). Isolate CG-T-63, CS-P-6, CG-P-65, and CS-P-20 were identified as *Enterobacter mori*, *E. bugandensis*, *E. asburiae* and *E. cloacae*, respectively. The prevalence of the isolates identified from the intestinal region supported the previous studies (Geethanjali and Anitha 2011, Romero *et al.* 2014, Kavitha *et al.* 2018, Abedin *et al.* 2020, Riaz *et al.* 2022, Zhai *et al.* 2023).

The phylogenetic tree was constructed based on neighbor joining method using the 16S rRNA gene sequences of seven isolates (Fig. 3). According to the phylogenetic grouping, strains with similar sequences were clustered in the same group and considered close relatives. The phylogenetic tree showed that the Gram-positive and Gram-negative isolates were originated from the same ancestor by forming two main branches consisting of *Bacillus* sp. strain MBIA2.42 and *B. subtilis* strain RYBGJ1 in one and *Acinetobacter baumannii* strain AB055 and a subgroup of *Enterobacter bugandensis* strain LSBU1 EV1, *E. mori* strain NAC52, *E. cloacae* strain

Table 1. Physiological characteristics of bacterial isolates.

Isolate No.	Catalase	Oxidase	Deep glucose agar	Motility	MR	VP	Starch hydrolysis	Citrate utilization	Propionate utilization	Nitrate reduction	Levan	Indole	Lecithinase	Lipase	Protease	Tyrosine degradation	Acid production			
																	D-glucose	D-Xylose	D-mannitol	L-arabinose
CS-P-4	+	–	FA	+	+	+	+	+	+	+(w)	+	+	+	+	+	–	+	+	+	+
CS-P-23	+	–	FA	+	–	+	+	+	–	+	+	+	+	–	+	–	+	+	+	+
CS-P-6	+	–	FA	+	–	+	–	–	+(w)	+	+	+(w)	+	+	+	+	–	–	+	+
CS-P-21	+	–	A	–	–	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+
CS-P-20	+	–	FA	+	+	+	–	–	+(w)	+(w)	–	–	–	+	+	–	–	+	–	+
CS-P-16	+	–	FA	+	+	+	–	+	+	+	+	–	+	+	+	–	+	+	+	+
CS-E-54	+	–	FA	+	–	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+
CG-P-41	+	–	FA	–	–	+	+	–	–	+	+	+(w)	+	+	+	–	+	+	+	+
CT-P-28	+	–	FA	+	+	–	+	+	–	+	–	+(w)	–	–	–	–	+	+	+	–
CG-P-38	+	–	FA	+	+	+	–	–	+	+	–	+	+	+	+	+	+	+	+	–
CG-P-44	+	–	FA	+	+	+	+	+	–	+	+	–	+	+	+	–	+	+	+	+
CT-C-34	+	–	FA	+	–	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+
CT-E-50	+	–	FA	–	+	+	–	–	–	+	–	+	+	+	+	+	+	+	+	+
CG-E-53	+	–	FA	+	+	+	–	+	+	+	+	–	+	+	+	–	+	+	+	+(w)
CT-E-52	+	–	FA	–	–	+	–	–	–	+	+	+(w)	+	+	+	+	+	+	+	+
CG-T-63	+	–	FA	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
CT-T-62	+	–	FA	+	–	–	–	–	+	+	+	+	–	+	–	+	+	+	+	+
CT-P-24	+	–	FA	+	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+	+
CT-P-26	+	–	FA	+	+	+	+	+	–	+(w)	+	+	–	+	+	+	+	+	+	+
CT-E-51	+	–	FA	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–
CT-C-36	+	–	FA	+	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	+
CT-C-35	+	–	FA	+	+	+	+	–	+	+	+	+	–	–	–	+	+	+	+	+
CG-P-37	+	–	FA	+	+	+	–	+	–	+	+	+	+	+	+	+	+	+	+	–
CG-P-65	+	–	FA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates positive result, – indicates negative result, A = aerobic, FA = facultative anaerobe and w = week.

D3BWTRM2 and *E. asburiae* strain WL115 in another. Since the members of the subgroup are from the same genus, they clustered together (Fig. 3).

Table 2. Identification of bacterial isolates of *Channa* spp..

Isolate	Gram reaction	Isolate	Gram reaction
Gram-positive			
CG-P-41	*Bacillus* sp.	CG-P-38	*B. subtilis*
CT-P-28	*B. subtilis*	CG-P-44	*B. licheniformes*
CS-P-4	*B. subtilis*	CT-C-34	*B. subtilis*
CS-P-23	*B. subtilis*		
Gram-negative			
CT-E-50	*Citrobacter diversus*	CT-C-36	*C. diversus*
CG-E-53	*Kluyvera cryocresens*	CT-C-35	*Pseudomonas aeruginosa*
CT-E-52	*Enterobacter* sp.	CS-P-6	*Enterobacter* sp.
CG-T-63	*Enterobacter* sp.	CG-P-37	*Citrobacter* sp.
CT-T-62	*Vibrio parahaemolyticus*	CS-P-21	*Acinetobacter* sp.
CT-P-24	*Citrobacter diversus*	CS-P-16	*P. aeruginosa*
CT-P-26	*Enterobacter* sp.	CG-P-65	*E. asburiae*
CS-P-20	*E. cloacae*	CS-E-54	*K. cryocresens*
CT-E-51	*Citrobacter* sp.		

Table 3. Sequence based identification of the selected bacterial isolates.

Isolate No.	Name of bacteria	Accession No.	E value	Identity
CS-P-6	*Enterobacter bugandensis* strain LSBU1 EV1	MN394114.1	0.0	99.57
CG-T-63	*E. mori* strain NAC52	MK872337.1	0.0	96.76
CS-P-20	*E. cloacae* strain D3BWTRM2	MH985217.1	0.0	97.54
CG-P-65	*E. asburiae* strain WL115	OK037550.1	0.0	98.07
CS-P-21	*Acinetobacter baumannii* strain AB055	KX955265.1	0.0	98.55
CG-P-41	*Bacillus sp.* strain MBIA2.42	KM438487.1	0.0	95.94
CS-P-4	*B. subtilis* strain RYBGJ1	MN721463.1	0.0	97.93

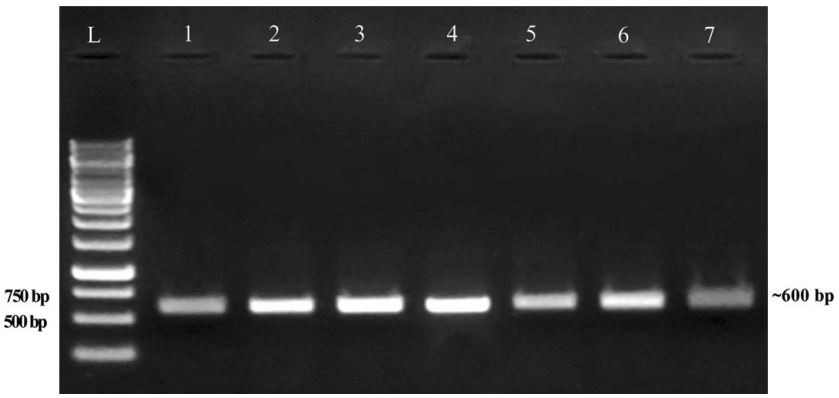

Fig. 2. Gel photographs show amplification of 16S rRNA gene of seven isolates. L-denotes molecular markers and numbers denotes the bacterial isolates.

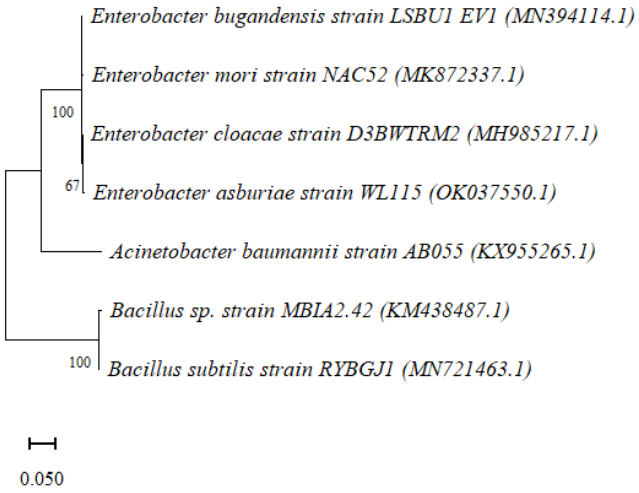

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA sequence.

Most bacterial isolates showed growth response at 30°C and 40°C and minimum or no growth was observed at 5 and 60°C indicating that that gut microbiome of fish prefers warm environment for their growth (Fig. 4). Since fish are poikilothermic animals, physiological stress may facilitate the proliferation of opportunistic bacteria in the gut by disrupting the balance (Wendling and Wegner 2013, Ghosh *et al.* 2022). However, isolate CS-P-20 showed no growth at 40°C, while highest growth was observed by CG-P-41 at 30°C and 40°C (Fig. 4). Maximum growth was also observed by the isolates CS-P-6 at pH 4.5, CG-T-63 at pH 6.5 and CG-E-53 at pH 8.5, respectively (Fig. 5). Furthermore, rest of the isolates showed a moderate growth in the tested pH indicating that the intestinal microbial community can grow well in slightly acidic or alkaline conditions.

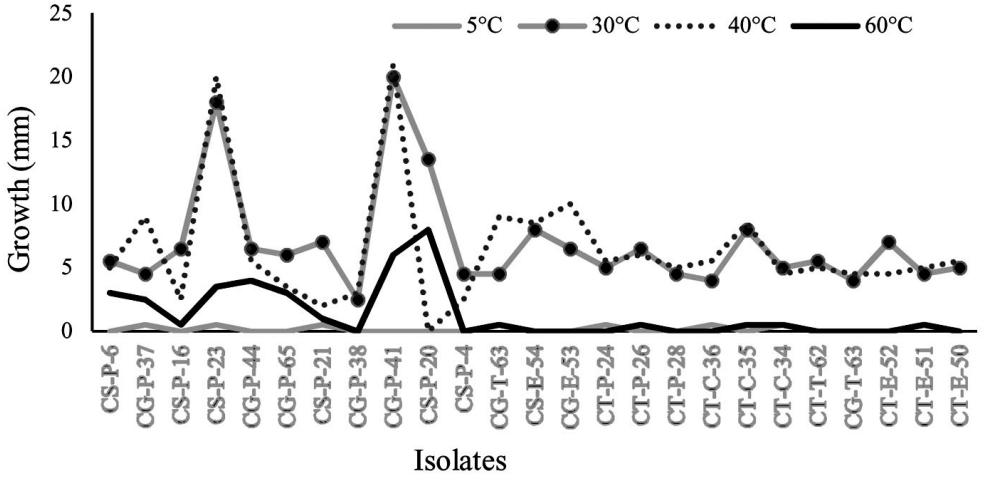

Fig. 4. Growth of bacterial isolates at different temperatures.

All the bacteria were resistant to cefixime and neomycin. *Enterobacter* spp. and *Pseudomonas aeruginosa* showed higher resistance to the eight antibiotics up to 100%. All the isolates showed sensitivity to ofloxacin up to 100% apart from *Citrobacter* spp. (60%) (Table 4). Since the antibiotic ofloxacin is categorized under the group fluoroquinolones, according to Ashiru *et al.* (2011), antibiotics in the fluoroquinolone class could be used to treat freshwater fish species.

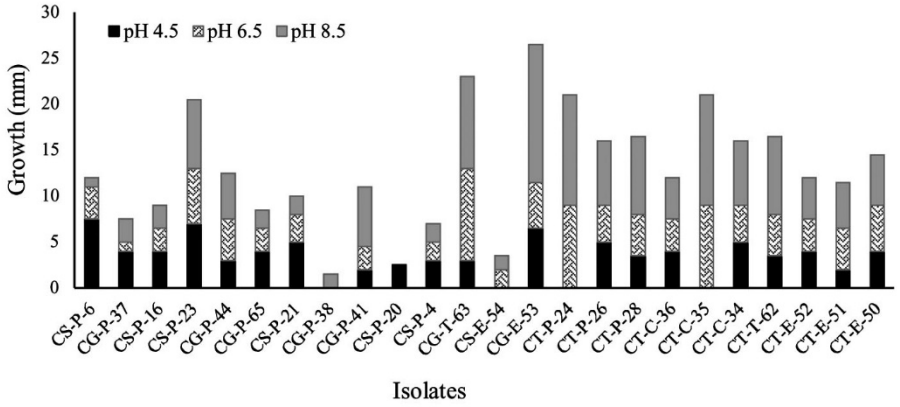

Fig. 5. Growth of bacterial isolates at different pH.

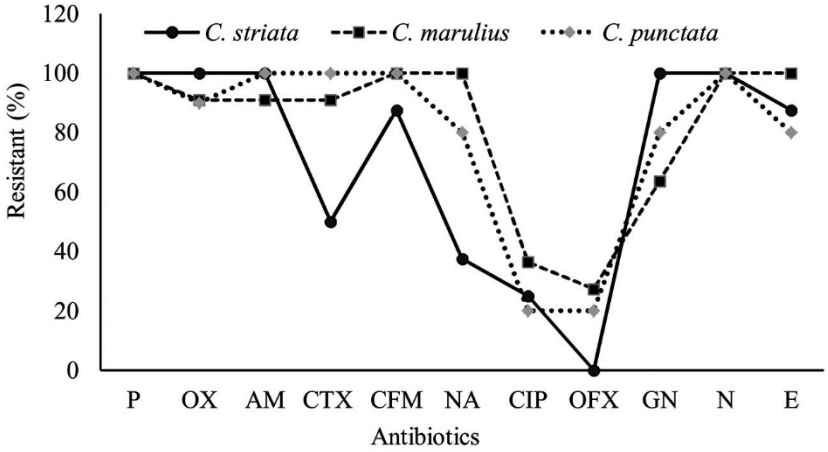

Fig. 6. Resistance of **bacterial** isolates of ***Channa*** spp. against different antibiotics.

The percentage of the gut bacterial isolates showing antibiotic resistance pattern were also observed in the three species of Channidae family (Fig. 6). The study revealed that the bacterial isolates of *C. punctata* showed maximum resistance to nine antibiotics in comparison to *C. marulius* for eight and *C. striata* for seven antibiotics, respectively, where the minimum value was counted as 80% (Fig. 6). The possibility of the gut bacteria of *C. punctata* showing greater resistance to antibiotics might be due to feeding habit and anthropic pollution in the waterbody (Laborda *et al.* 2022).

Table 4. Antibiogram profile of bacterial isolates.

Antibiotics	Conc. (µG)	*Bacillus* spp.			*Enterobacter* spp.			*Citrobacter* spp.			*Kluyvera cryocresens*			*Pseudomonas aeruginosa*		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Penicillin	10	85.7	14.3	0	100	0	0	100	0	0	100	0	0	100	0	0
Oxacillin	1	71.4	14.3	14.3	100	0	0	100	0	0	100	0	0	100	0	0
Ampicillin	10	71.4	28.6	0	100	0	0	100	0	0	100	0	0	100	0	0
Cefotaxime	30	85.7	14.3	0	100	0	0	100	0	0	50	50	0	100	0	0
Cefixime	5	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Nalidixic acid	30	85.7	14.3	0	83.3	16.7	0	80	0	20	100	0	0	50	50	0
Ciprofloxacin	5	42.9	42.9	14.3	33.3	33.3	33.3	20	0	80	50	50	0	50	50	0
Ofloxacin	5	0	0	100	0	0	100	20	20	60	0	0	100	0	0	100
Gentamycin	10	71.4	14.3	14.3	100	0	0	80	0	20	100	0	0	100	0	0
Neomycin	30	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Erythromycin	15	85.7	14.3	0	100	0	0	60	40	0	50	50	0	100	0	0

R = Resistant, I = Intermediate and S = Susceptible.

Since the specimens were collected from a wild aquatic habitat, the prevalence of gut microbes being antibiotic resistant should be considerably low. However, the result of the tested antibiotics indicates a critical condition of Chinadi beel which can eventually deteriorate the health of the inhabited aquatic organisms. This study can be used as baseline data regarding fish gut bacterial population in Chinadi beel and should be further investigated since no records have been found yet.

Acknowledgments

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