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ANTIBIOTIC SENSITIVITY OF BACTERIA ISOLATED FROM NILE TILAPIA (*Oreochromis niloticus*) FISH FROM GAZIPUR FISH MARKET

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ABSTRACT

Nile tilapia *(Oreochromis niloticus*) is extensively cultured in Bangladesh. The objective of this study was to detect the health hazard microbes (*Vibrio cholerae, Salmonella* spp., *Escherichia coli* and other pathogenic bacteria) in Nile tilapia fish and their sensitivity against the antibiotics. Total plate count (TPC) of bacteria, *E. coli* count, presence/absence of *Salmonella*, *Vibrio* and antibiotic sensitivity test of the isolated bacteria were done by the standard microbiological method. The highest TPC was 4.995 Log 10 CFU/g, and the lowest was 4.822 Log10 CFU/g**.** *E. coli* ranged from 2.462 Log10 CFU/g to 2.903 Log10 CFU/g. The TPC value showed insignificant-variations and almost all were within the acceptable limit range. No *Salmonella* and *Vibrio cholerae* were found. *Escherichia coli* were found in seven samples, *Leclercia adecarboxylata* were found in six samples, and *P. alimentorum* was found in six samples. Seven antibiotics were used for antibiotic resistance testing, and isolated bacteria mostly showed resistance toward ampicillin, and only four isolates showed resistance to nitrofurantoin and oxytetracycline. The highest sensitivity found for ciprofloxacin. Results of the study indicate that pathogenic bacteria are present in retail tilapia fish, and some are resistant to antibiotics. Consumers in Gazipur are at risk regarding for antibiotic resistance of tilapia.

Keywords: *Oreochromis niloticus*, Total plate count, *Escherichia coli, Leclercia adecarboxylata*, *Proteus alimentorum*, antibiotic sensitivity

Introduction

Bangladesh has become the third-largest aquaculture-producing country globally (FAO 2018). Over the last three decades, tilapia (*Oreochromis niloticus*) production has been significantly developed worldwide. It is considered one of the most productive and internationally traded food fish globally (Hernández *et al.* 2013). In Bangladesh, tilapia is mainly preferred in aquaculture because of its good resistance to poor water quality and disease, tolerance to a wide range of environmental conditions, ability to convert the organic and domestic waste efficiently into high-quality protein, rapid growth rate and tasty flavor (Siddik *et al.* 2007).

However, microbiological food safety is a primary concern nowadays. Food-borne pathogenic bacteria, including *Salmonella enterica*, enteropathogenic *Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica*, and *Klebsiella pneumonia,* were identified from fresh Nile tilapia in Kenyan freshwater fish chains (David *et al*. 2009). Wound infections with *V. vulnificus* have been reported in fishers and other individuals while handling certain types of fish, including *tilapia* species (Calif *et al*. 2003).

Vibrio is gram-negative, ubiquitous in marine and estuarine ecosystems and aquaculture farms, and comprises one of the significant microbiota of these ecosystems. Vibriosis, caused by infection by *Vibrio* spp, is one of the most prevalent diseases in fishes and other aquaculture-reared organisms and is widely responsible for mortality in sophisticated aquaculture systems worldwide (Chen *et al.* 2000).

Worldwide, *Salmonella* is the leading reason of bacteria-associated disease outbreaks. This species is found in different countries with different sources such as farm to retail store, fish skin to the intestine, both in raw fish and

fishery products and present in imported and domestic fishes (Sheng and Wang 2020). *Salmonella* is responsible for salmonellosis disease, and symptoms include abdominal pain, nausea, diarrhea, vomiting and fever.

Different antibiotics are used in tilapia culture to keep healthy fish free from diseases. Common antibiotic groups used in aquaculture are oxytetracycline, chloramphenicol, trimethoprim-sulphamethoxazole, nitrofurantoin, nalidixic acid, and ampicillin (Soto-Rodríguez *et al.* 2015). Developing countries have no regulations on antibiotic use, which inspires indiscriminate use of antibiotics. Antibiotics application at sub-therapeutic (low) doses or for a short period can contribute to the development of antibiotic resistance in fish. Antibiotic resistance among fish bacteria results from excessive use of antibiotics (Cabello *et al.* 2013). Integrated culture inspires more antibiotic-resistant bacteria and acts as a storehouse than the traditional one. In conjugation, transformation and transduction processes, horizontal gene transfer occurs, and vertical transfer occurs via reproduction. Contaminated animal products and farm workers may transfer resistant bacteria to humans. Thus, it is evident that in each of these cases, several highly complex environmental conditions can lead to the transfer of bacterial resistance from aquaculture practices to humans and ecosystems through the food web.

Though tilapia is a very common and popular fish in Bangladesh, the microbiological status of retail market tilapia and their antibiotic resistance status in Bangladesh has not been reported yet. Hence, the purpose of this study was to investigate the total plate count, occurrence of *Salmonella, Vibrio Cholerae, Escherichia coli*, other pathogenic bacteria and antibiotic susceptibility of isolated bacteria from fresh tilapia fish from Chowrasta fish markets of Gazipur of Bangladesh.

Materials and Methods

Sample collection: A total of 45 fresh tilapia (*Oreochromis niloticus*) fish samples were collected in three batches (each batch contain 15 sample) from the Chowrasta fish market of the Gazipur district. The samples were collected from March 2019 to June 2019, and the collected samples were immediately iced and transported to the Microbiology Laboratory at the Department of Fisheries Technology of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU).

Determination of total plate count (TPC), *Escherichia coli, Salmonella* and other pathogenic bacteria: The total bacterial load and determination of other pathogenic bacteria were estimated using the method described in the Bacteriological Analytical Manual (Maturin and Peeler 2001). *Escherichia coli* determination was followed by the Petri film method (3M™ Petrifilm™ *E. coli*). *Salmonella* testing followed the standard Bacteriological Analytical Manual (Andrews *et al*. 2000). *Vibrio cholerae* testing followed the standard Bacteriological Analytical Manual (Kaysner 2004).

Molecular identification of bacteria: From pure culture stock, bacterial colonies were transferred into a nutrient broth (Himedia, India) and incubated for 48 h at 28°C in a shaker incubator (120 rpm) (Hannan *et al.* 2019). A GeneJET Genomic DNA Purification Kit (Thermo scientific, #K0721, USA) was used for DNA extraction. The polymerase chain reaction (PCR) with universal primer sets was used for amplification. The purified PCR products (Fig. 1) with sequencing primer were sent to the National Institute of Biotechnology, Savar, Dhaka, to sequence the 16S rRNA gene.

Antibiotic sensitivity test: The Kirby-Bauer disc diffusion method was used for the antibiotic sensitivity test (Hudzicki 2009). Seven antibiotics were used: ampicillin (25 µg/disc), gentamycin (10 µg/ disc), chloramphenicol (10 µg/disc), oxytetracycline (30 µg/disc), nitrofurantoin (300 µg/disc), levofloxacin (5µg/ disc), and Ciprofloxacin (5 µg/ disc). The zone diameter around the disc was measured after incubation according to CLSI (Clinical and Laboratory Standards Institute 2018).

Statistical analysis: One-way ANOVA was applied to determine the significant difference in total plate count among batches. A $p < 0.05$ was set to indicate statistical significance. The Bioresource in Adult Infectious Diseases (BIOAD) and BLAST (Basic Local Alignment Search Tool) were used for analyzing sequence data.

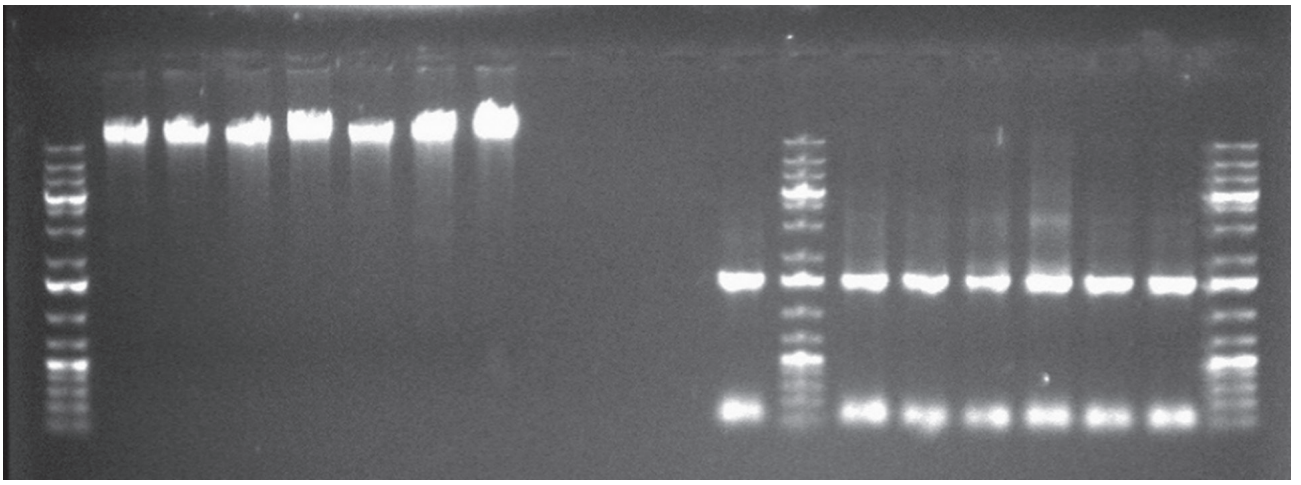


Figure 1. PCR amplification of 16S rRNA gene of the bacteria isolates

Results and Discussion

No *Salmonella* and *Vibrio cholerae* were found in any sample. *Salmonella* was absent in the present study possibly due to the cooled condition maintained by the fish seller. In contrast, *Vibrio cholera* was not found in the samples because *V. cholerae* is ubiquitous on marine and brackish water bodies. Therefore, it was absent in freshwater tilapia ponds in the Mymensingh region (Faruk 2008). However, *Escherichia coli* were found in seven samples. For example, *Leclercia adecarboxylata* and *Proteus alimentorum* was found in six samples (Table 2).

The total plate count (TPC) of fish varied among the species. The highest TPC was 4.995 Log 10 CFU/g, and the lowest was 4.822 Log 10 CFU /g, a significant difference ($p < 0.05$) was found among the fish species. Variation in the bacterial load may be attributed to bacterial initial contamination level in fish skin and the multiplication of organisms favored at the storage condition. Besides, handling during transportation and personal hygiene during filleting may be the reason for variation in TPC value. The TPC in tilapia was found to be within the acceptable limit. In Bangladesh, Begum *et al.* (2010) found the total plate count in tilapia (*Oreochromis niloticus)* muscle from 5.2 log cfu/g to 6.7 log cfu/g, and Mandal *et al.* (2009) found 5.3 Log cfu/g to 5.9 Log cfu/g. The total plate count of bacteria obtained in this study was found to vary from the findings of other researchers. Because the number of bacteria can vary due to raw material conditions, processing methods, surrounding environmental conditions, and cross-contamination.

E. coli was found in tilapia fish of all three batches, ranging from 2.5 Log10 CFU/g to 2.9 Log10 CFU/g (Table 1). Only seven samples contained E. *coli* (36.84% of isolates) (Table 2). In Bangladesh, Nile tilapia was sampled from Satkhira district and Farmgate fish market where *Escherichia coli* was 240 MPN/g and 1350 MPN/g (Mandal *et al*. 2009). Begum *et al*. (2010) found total fecal coliform count in Tilapia (*Oreochromis niloticus)* muscle ranging 2.3 to 15 MPN/g in Bangladesh. The previous studies suggest that the presence of *E. coli* in Nile tilapia may be due to the fecal contamination during the culture period, storage, transportation, unhygienic marketing, and lack of hygienic handling or poor sanitary conditions prevailing at the landing center (Mandal *et al*. 2009). The water in Bangladesh can be contaminated during the

Table1. Total plate count (TPC) and *E. coli* count isolated from tilapia fish.

Bacteria	Minimum	Maximum	Mean
TPC (Log 10 CFU/g)	4.822	4.995	4.93± 0.057
E. coli (Log 10CFU /g)	2.462	2.903	2.613 ± 0.200

rainy season from various sources, including poor waste management systems, poor sanitary conditions, pets, or poultry from nearby farms (Mandal *et al*. 2009). Fish markets can also be a source as fish market conditions in Bangladesh are not hygienic. This result indicates that tilapia was contaminated with *E. coli*, indicating poor sanitation practices in the supply chain.

Around 31.6 % of isolates were *Proteus alimentorum* found in tilapia fish collected from the Chowrasta fish market (Table 2). Human intestines are considered a reservoir of *Proteus* spp. *Proteus* spp. is present in soil or water habitats and often regarded as an indicator of fecal pollution (Dai *et al.* 2018). The fish might be cross-contaminated with *P. alimentorum* from the soil, water, or animals. Besides, it may come from contaminated feed materials. Other possible reasons of contamination might be human feces during culture, transportation, washing, handling and retail sale. This organism can cause urinary tract infections, wounds and burns infections, abscesses, respiratory tract infections, bacteremia, meningitis, intestine colonization (diarrhea), nosocomial infections, and rheumatoid arthritis (Siwińska *et al*. 2019). Therefore, consumption of tilapia contaminated with *P. alimentorum* is unsafe if not properly cooked or undercooked. The possible causes of presence of this bacteria in tilapia fish might be the improper handling of fish or unhygienic water conditions in which fishes were cultured or unhygienic handling and transportation.

Around 31.6 % of isolates were *Leclercia adecarboxylata* (Table 2). *L. adecarboxylata* is previously known as *Escherichia adecarboxylata*, distributed in nature ubiquitously (Spiegelhauer *et al*. 2019). As it is widely found in food, water, and other environmental sources, its presence in fish is not unusual. Multidrug-resistant *L. adecarboxylata* was found in the river and Lake of Dhaka city (Haque *et al*. 2015). This species is also found in the oral cavity of shark in Brazil, and this information link this species to the marine and water environment (Interaminense *et al*. 2010). However, the presence of this species in tilapia fish or other fishes in Bangladesh has not been reported yet. People who already suffer from primary diseases such as cancer, leukemia, cirrhosis etc., can easily affect by pneumonia, septicemia, and wound infections through this pathogen (Matsuura and Sugiyama 2018). Adedeji and Onwenefah (2013) reported that *L. adecarboxylata* was found in the stomach of cultured catfish fed with poultry hatchery waste in Ibadan Southwestern Nigeria. Therefore, the feed could be a possible source of *L. adecarboxylata* contamination in tilapia analyzed in this study. Emerging opportunistic human pathogen *L. adecarboxylata* was also detected in Mossambic tilapia fish collected from water bodies impacted by urban waste bacteria (Stock *et al*. 2004). The possible sources of *L. adecarboxylata* might be the unhygienic handling and cross-contamination as significantly less hygiene is maintained in fish markets in Bangladesh. If fish are stored in cool conditions and maintain hygiene conditions in every step from processing to marketing, then the presence of this harmful bacteria can be minimized.

Proteus alimentorum showed the most sensitivity against ciprofloxacin and resistance against ampicillin (Table 3). No resistance was observed against ciprofloxacin,

Table 2. Different type's bacteria isolated from tilapia fish

SL No	Bacterial Isolates	Frequency No (%)	1st batch	2nd batch	3rd batch
1.	*Salmonella*	0 (0)	-	-	-
2.	*Vibrio cholera*	0(0)	-	-	-
3.	*Escherichia coli*	7(36.8)	+	+	+
4.	*Leclercia adecarboxylata*	6(31.6)	+	+	-
5.	*Proteus alimentorum*	6 (31.6)	-	-	+
	Total	19 (100)			

Table 3. Antibiotic susceptibility pattern of *P. alimentorum*

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	0	0	0	0	6	100
Ciprofloxacin	6	100	0	0	0	0
Gentamycin	4	66.7	2	33.3	0	0
Nitrofuran	2	33.3	2	33.3	2	33.3
Levofloxacin	2	33.3	4	66.7	0	0
Chloramphenicol	3	50	3	50	0	0
Oxytetracycline	3	50	2	33.3	1	16.7

gentamycin, levofloxacin and chloramphenicol. These findings were somehow in agreement with the resistance profile report of *P. alimentorum*. The previous study showed *P. alimentorum* was susceptible to ciprofloxacin, gentamicin, tetracycline and chloramphenicol (Dai *et al.* 2018). In the present research, *P. alimentorum* showed 100% sensitivity to ciprofloxacin and 100% resistance to ampicillin. However, Dai *et al.* (2018) reported *P. alimentorum* showed intermediate resistance to ampicillin.

L. adecarboxylata showed the most sensitivity to ciprofloxacin (100%) and the most resistance to ampicillin (Table 4). The finding of this study supports the previous reults (Matsuura and Sugiyama 2018) as *L. adecarboxylata* is very susceptible to many antibiotics (tetracyclines, nitrofurantoin, aminoglycosides, chloramphenicol, azithromycin) and naturally resistant to erythromycin, streptogramins, lincosamides, fusidic acid, penicillin G, clarithromycin, oxacillin, roxithromycin, ketolides, linezolid, glycopeptides, rifampicin, and fosfomycin (Matsuura and Sugiyama 2018). Except for intrinsic resistance, Yao *et al*. (2011) found the resistance of *L. adecarboxylata* (isolated from pig) was found to aminoglycosides, quinolones and trimethoprim-sulfamethoxazole. On the other hand, multidrug-resistant *L. adecarboxylata* was isolated from the river and lake of Dhaka city, where resistance showed to ampicillin, amoxicillin, ciprofloxacin, ceftriaxone, ceftazidime, aztreonam and trimethoprim-sulfamethoxazole (Haque *et al*. 2015).

Table 4. Antibiotic susceptibility pattern of *Leclercia adecarboxylata*

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	0	0	0	0	6	100
Ciprofloxacin	6	100	0	0	0	0
Gentamycin	3	50	3	50	0	0
Nitrofuran	4	66.7	2	33.3	0	0
Levofloxacin	5	83.3	1	16.7	0	0
Chloramphenicol	3	50	3	50	0	0
Oxytetracycline	2	33.3	3	50	1	16.7

Table 5. Antibiotic susceptibility pattern of *E. coli* isolates

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	0	0	0	0	7	100
Ciprofloxacin	7	100	0	0	0	0
Gentamycin	4	57.14	3	42.85	0	0
Nitrofuran	3	42.85	4	57.14	0	0
Levofloxacin	2	28.57	5	71.42	0	0
Chloramphenicol	5	71.42	2	28.57	0	0
Oxytetracycline	4	57.14	2	28.57	1	14.28

E. coli also showed the most resistance to ampicillin (Table 5). Interestingly *E coli* showed resistance to oxytetracycline (14.3 %). The previous report of resistance of *E coli* isolated from seafood is similar for ampicillin and tetracycline; however report also shows resistance toward chloramphenicol, ciprofloxacin and gentamycin (Ryu *et al*. 2012).

Although ampicillin is used to treat infections of the respiratory tract, skin and urinary tract (Duran and Marshall 2005), but no isolates showed sensitivity to ampicillin. In this research, all isolates were sensitive and in the intermediate range for gentamycin and levofloxacin. For *Proteus* infection, farmers used gentamycin, thereore sensitivity for gentamicin is a good result for disease control.

Interestingly, banned antimicrobial drugs (nitrofurantoin, chloramphenicol) showed sensitive characteristics for all isolates except for *P. alimentorum* (nitrofurantoin 33.3%). Oxytetracycline was also very effective but all species (*P. alimentorum, L. adecarboxylata* and *E. coli*) showed resistance to oxytetracycline. It indicates that indiscriminate use of these specific antibiotics may cause resistance. As oxytetracycline is used widely in fish farming, bacteria isolates in intermediate and resistance zones are not surprising.

Disease outbreaks in fish farming are widespread in Bangladesh. Antibiotics are used to prevent and treat diseases, but unregulated and indiscriminately uses are observed in many parts of the world, especially in Asia and Africa. Faruque *et al*. (2008) reported that most of the farmers could not recognize the actual disease. Failure to recognize the disease is another cause for the development of antibiotic resistance as incorrect antibiotics are used.

Conclusions

This study was conducted to investigate the presence of pathogenic bacteria in tilapia fish and the antibiotic susceptibility of isolated bacteria. Some bacteria are natural microflora in tilapia farms, but the presence of harmful bacteria indicated that fecal contamination occurred at some point in the supply chain. The consumers in Bangladesh are at risk regarding food safety if proper handling is not followed and fish farmers and people could face additional disease challenges due to the development of resistant bacterial strains.

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