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Antibiotic sensitivity of the bacteria isolated from pangas (*Pangasius hypophthalmus)* fish of the retail fish market of Gazipur, Bangladesh

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Abstract

Food safety associated with microbiological quality has become a critical issue worldwide. The current study was undertaken to detect the health hazard microbes (Total plate count, *Vibrio cholerae*, *Salmonella* spp., *Escherichia coli* and other pathogenic bacteria) in pangas fish and their sensitivity against the antibiotic. Fresh pangas fish were collected from different fish markets in Gazipur (Chowrasta, Shimultoli and Joydebpur fish market) district of Bangladesh. Total plate count (TPC) of bacteria, *E. coli,* other pathogenic bacteria and presence/absence test of *Salmonella* and *V. cholerae* were tested and the antibiotic resistance pattern of isolated bacteria was observed. The highest TPC was 5.22 $\log_{10}$ CFU/g from Chowrasta fish market and the lowest was 4.795 $\log_{10}$ CFU/g from Shimultoli fish market. *Escherichia coli* ranged from 2 $\log_{10}$ CFU/g (Chowrasta fish market) to 2.698 $\log_{10}$ CFU/g (Shimultoli fish market). No *Salmonella* and *V. cholerae* were found, *E. coli* was found in eight samples, *Leclercia adecarboxylata* (accession no. MN625850, MN625851) was found in five samples, and *Aeromonas caviae* (accession no.MN625853) were found in eight samples. *Escherichia coli* was found in pangas fish collected from all markets, ranges were 2 $\log_{10}$ CFU/g (Chowrasta fish market) to 2.698 $\log_{10}$ CFU/g (Shimultoli fish market) and all of them were within the range of acceptable limit. Seven antibiotics, namely ampicillin (25 µg/disc), gentamycin (10 µg/disc), chloramphenicol (10 µg/disc), oxytetracycline (30 µg/ disc), nitrofurantoin (300 µg/ disc), levofloxacin (5 µg/ disc), and ciprofloxacin (5 µg/disc) were used for antibiotic resistance testing. Isolated bacteria mostly showed resistance toward ampicillin, and only two isolates showed resistance to nitrofurantoin and oxytetracycline. The highest sensitivity showed for ciprofloxacin. The results of the study indicated that pathogenic bacteria present in retail pangas fish, and some are resistant to antibiotics and consumers in Bangladesh are at risk for food safety. If proper handling is not followed, fish farmers and people could face additional disease challenges due to the development of resistant bacterial strains.

1. Introduction

Microbiological food safety is a primary concern nowadays. Microorganisms, namely bacteria are natural microflora in fish, and some bacteria are present due to cross-contamination during poor handling. Illness due to the consumption of contaminated food is a critical problem worldwide. Food-borne disease outbreaks from shrimp and fish are well-documented (Alday-Sanz, 2010). The presence of foodborne pathogens in fish depends on the microbiological status of the surrounding environment and water, fishing method, storage situation and cross-contamination through agricultural sources, animals, and humans (Feldhusen, 2000; Davies *et al*., 2001; Hosseini *et al*., 2004). The total number of bacteria more than 1 x10^5 CFU/g in fish flesh is not acceptable for human consumption (ICMSF, 1986). On the other hand, fish contain bacterial pathogens causing human diseases such as *Vibrio cholerae*, *Vibrio vulnificus*,

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Vibrio parahaemolyticus, pathogenic *Escherichia coli*, *Salmonella* spp., *Aeromonas* spp., *Streptococcus iniae, Listeria monocytogenes*, *Plesiomonas shigelloides, Clostridium botulinum*, *Mycobacterium* spp., *Campylobacter jejuni, Edwardsiella tarda* and *Clostridium perfringens (*Novotny *et al*., 2004). Pathogens transmitted from either the aquatic environment or fish to humans are usual. It can occur by contacting an infected person in a related environment, and transmission can rely on the season, food habits of an individual person, handling of fish and cross-contamination (Novotny *et al*., 2004). The microorganisms are naturally present in food but they became critical due to their opportunistic pathogenic characteristics and pathogenic characteristics in nature (Mhango *et al*., 2010).

Worldwide, *Salmonella* is the leading bacterial reason of fish-associated disease outbreaks (Sheng and Wang, 2021). *Salmonella* is a Gram-negative bacteria of the Enterobacteriaceae family. It is not a natural inhabitant of fish, and it is introduced to fish by cross-contamination through contaminated water and improper handling (Fernandes *et al*., 2018). This species is found in different countries with different sources such as farm to retail stores, fish skin to the intestine, raw fish and fishery products, and imported and domestic fishes (Sheng and Wang, 2021). *Salmonella* is responsible for salmonellosis disease, and symptoms include abdominal pain, nausea, diarrhea, vomiting and fever (Ray and Bhunia, 2007; Jajere, 2019).

Vibrio is a natural microflora of the aquatic environment and is common in pangas farms, causing disease (Gopal *et al*., 2005; Parven *et al*., 2020). Globally pathogenic *Vibrio* is a public health concern for the fish consumer, and the presence of any pathogenic *Vibrio* is the reason for rejection in international trade and import bans (WHO, 2001). Among *Vibrio* spp, the most important human pathogens are *V. cholerae, V. vulnificus*, and *V. parahaemolyticus* (Gopal *et al*., 2005). *V. cholerae* is of most concern as it is the reason cholera makes it globally a public threat. The serotypes of *V. cholera* O1 and O139 are responsible for causing cholera by producing enterotoxin, and other non-O1 and non-O139 are not so harmful, responsible for septicemia and mild gastroenteritis (Gopal *et al*., 2005). Symptoms of this bacterium infection include hypoglycemia in children, rapid dehydration, renal failure, acidosis and circulatory collapse.

Escherichia coli indicates fecal contamination and causes health problems like diarrhea, kidney and bladder infections, dysentery, and hemolytic uremic syndrome, depending on the strains (Ray and Bhunia, 2007). There are six types of diarrheagenic *E. coli*: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), diffusely adherent (DAEC), enteropathogenic (EPEC) and Shiga toxin-producing (STEC) *E. coli* (Costa, 2013). Due to the poor sanitary condition of the country, the aquatic habitats of Bangladesh are possibly heavily contaminated with fecal coliform bacteria. Fishes that live in these polluted habitats can easily intake harmful bacteria while feeding along with contaminated aquatic foods. Fish of good quality should not exceed fecal coliforms 10/g and staphylococci 100/g (FAO, 1997). Excluding the above bacteria, *Aeromonas* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Flavobacterium* spp. and *Edwardsiella* spp. were also found in diseased pangas fish (Abedin *et al*., 2020).

Pangasius hypophthalmus, popularly known as ‘thai pangas’, was introduced to Bangladesh from Thailand in 1990 and has become a popular fish species like other South Asian countries due to its rapid growth rate, low mortality, large size and year-round production (Parven *et al*., 2020; Faruk, 2008). Farming of Thai pangas is a significant component of aquaculture in Bangladesh, and in 2016, total production was 494,357 tons, accounting for 29% of the total farmed fish supply in the country (Department of Fisheries Bangladesh, 2017). Considering the great potential of pangas farming and its vital role in national food security, its production should be increased to ensure good quality.

Significant economic losses occur due to fish mortality in pangas farms by disease, which is mainly associated with the genus *Aeromonas* spp., *Streptococcus* spp., *Vibrio* spp., *Flavobacterium* spp. and other pathogens (Abedin *et al*., 2020). For this reason, in the modern culture system, antibiotics are being employed prophylactically and therapeutically to protect fish from bacterial diseases and for growth promotion (Avsever *et al*., 2010). However, the incidence of antibiotic-resistant bacteria is very dangerous for fish farms and the aquatic environment. According to the World Health Organization, antibiotic resistance is considered one of the crucial problems for human health (Bassetti *et al*., 2011). The most dangerous threat of drug-resistant bacteria in an aquatic environment is the potential transfer of a drug-resistant strain from the aquatic environment to the terrestrial environment, potentially affecting humans. Resistant bacterial strains can also be transferred to the human body by ingesting seafood that contains resistant bacteria (Gräslund and Bengtsson, 2001).

Identification and isolation of bacteria from diseased pangas fish and those bacterial sensitivities to antibiotics have already reported (Faruk, 2008; Abedin *et al*., 2020;

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Parven *et al*., 2020). However, the microbiological status of pangas found in the retail market and their antibiotic resistance status in Bangladesh have not been reported yet for food safety purposes. Hence, this study aimed to investigate the bacterial quantity, bacterial quality (occurrence of *Salmonella, V. cholerae, E. coli*, and other pathogenic bacteria) and antibiotic susceptibility of isolated bacteria from fresh pangas fish from different markets of Gazipur of Bangladesh.

2. Materials and methods

2.1 Sample collection

A total of forty-five Thai pangas (*Pangasius hypopthalmus*) fish samples were collected from Chowrasta, Shimultoli and Joydebpur fish markets of the Gazipur district. The average weight of the samples was from 1 to 1.2 kg. 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 BSMRAU. In the laboratory, each fish sample was cut into pieces with a sterile knife aseptically, and the flesh was packed in a zip lock bag and kept in a freezer at -20°C until analysis. Only flesh was used as consumers prefer to eat flesh generally.

2.2 Determination of total plate count

The bacterial load was estimated using the method described in the Bacteriological Analytical Manual (Maturin and Peeler, 2001). The aerobic plate count method was used to indicate the level of microorganisms in pangas. Accurately measured 25 g homogenized sample and 225 mL phosphate buffer saline (0.85% NaCl) were blended for 60 s. After the preparation of consecutive dilutions (10^{-1} to 10^{-4}), 1 mL of each dilution was transferred to petri dishes in duplicate. Around 15 mL plate count agar (Himedia, India) was poured into each plate and the incubation period was 24 hrs at 35°C. All plates were visually examined, and countable plates showing 25 to 250 colonies were selected and counted.

2.3 Determination of Escherichia coli

Escherichia coli determination followed the petrifilm method (3M™ Petrifilm™ *E. coli*). Aseptically, 25 g of pangas sample was blended with 225 mL sterile phosphate-buffered saline (PBS). The pH of the solution was adjusted to 6.6-7.2, and solutions were prepared up to 10^{-4} dilutions. Accurately measured 1 mL diluted sample was placed in the middle of the 3M petrifilm, the lid of the film was closed, and the 3M petrifilm spreader was placed on the petrifilm to spread the inoculum properly. Then, the spreader was removed and the plate was left for 1 min to permit the gel to form. After that, 3M petrifilm was incubated at 35°C for 24 hrs. Then the blue gas-forming colonies were counted as an indicator of *E. coli*.

2.4 Determination of Salmonella

Salmonella testing followed the standard Bacteriological Analytical Manual (Andrews *et al*., 2000). Aseptically 25 g of sample were blended with 225 mL sterile lactose broth (Himedia, India), and the homogenized mixture was incubated at 35°C for 24 hrs. Then 0.1 mL of the pre-enriched sample was mixed with 10 mL of Rappaport-Vassiliadis broth or RV broth, incubated at 42°C for 24 hrs and after incubation, a 3 mm loopful of RV broth was streaked on xylose lysine desoxycholate or XLD (Himedia, India) agar, incubated for 24 hrs at 35°C. After incubation, petri dishes were examined for the presence of salmonella colonies (pink colonies with or without black centers). The susceptive colonies were streaked on nutrient agar to isolate a single colony or pure culture. Incubation of the nutrient agar (NA) petri dish was done for 24 hrs at 35°C. For the confirmation test, triple sugar iron agar (TSI) (Himedia-M021) and lysine iron agar (LIA) (Himedia-M377) were used.

2.5 Determination of Vibrio cholerae

Vibrio cholerae testing followed the standard Bacteriological Analytical Manual (Kaysner, 2004). Aseptically 25 g of pangas sample and 225 mL alkaline peptone water were blended for 60 s and incubated at 35°C for 24 hrs. Thiosulfate Citrate Bile Salt Sucrose (TCBS) (Himedia, India) agar plates were used for streaking and incubated at 35°C for 24 hs. The isolates were routinely sub-cultured on NA plates and incubated at 37°C for 24 hrs.

2.6 Determination of other pathogenic bacteria

Serially diluted 100 µL of the diluted (10^{-3} to 10^{-7}) samples were spread on plate count agar (Himedia, India) and incubated at 37°C for 24 hrs. The isolates were systematically sub-cultured on nutrient agar, and stock cultures were maintained in NB (Nutrient Broth) supplemented with 10% glycerol and stored in a freezer at -80°C before molecular identification.

2.7 Molecular identification of bacteria

Molecular identification follows five steps namely, isolation of genomic DNA, DNA quality measurement by gel electrophoresis, amplification by PCR, purification of PCR sample and finally DNA sequencing of isolated bacteria (Hannan *et al*., 2019). Susceptive bacterial colonies were taken from pure culture stock, inoculated into a nutrient broth (Liofilchem), and

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incubated in a shaker incubator (120 rpm) at 28°C for 24 -48 hrs. After incubation, bacterial colonies were used for genomic DNA extraction. GeneJET Genomic DNA purification kit # K0721 (Thermo scientific) protocol was used for genomic DNA extraction. The purified DNA was found. Electrophoresis was used to check the DNA quality by comparing it with the 1 Kb plus DNA ladder marker (Thermo Fisher Scientific, USA). In the gel documentation system, the DNA band was observed under UV light (UVDI, Major Science).

The polymerase chain reaction (PCR) with universal primer sets was used for amplification (Table 1). The concentration of the PCR mixture is given in Table 2. The PCR thermocycler (2720 thermal cycler, Applied Biosystems) was used for amplification. The thermal profile of PCR was: the initial dilution step was set up for 5 min at 94°C, the denaturation step of 35 cycles was set up at 94°C for 1 min, annealing for 40 s at 57°C, and the extension step set up was for 1 min at 72°C, and final extension step was set up for 10 min at 72°C. After agarose gel electrophoresis, a gel documentation system visualized the amplicons under UV light (UVDI, Major Science).

The PCR product was purified using a commercial PCR Purification Kit (Thermo Scientific GeneJET PCR Purification Kit #K0701), and the purified PCR product was stored at -20°C for further use.

The purified PCR products with sequencing primer were sent to the National Institute of Biotechnology, Savar, Dhaka, to sequence the 16S rRNA gene. After getting sequencing results, they were searched using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/).

2.8 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 0.5 McFarland standard was used to check the visual density of bacteria. Accurately measured 50 µL individual isolates broth were transferred into iso sensitest agar media (ThermoFisher, USA), and then commercially prepared discs (Liofilchem, Italy and Himedia, India) were placed and incubated for 18 hrs at 35°C. The zone diameter around the disc was measured after incubation according to CLSI (Table 3) (Clinical and Laboratory Standards Institute, 2018).

2.9 Data analysis

One-way ANOVA was applied to determine the significant difference in total plate count among fish markets. A $p<0.05$ was set to indicate statistical significance. The BIOAD software and BLAST (Basic Local Alignment Search Tool) were used for analyzing sequence data (Rahman *et al*., 2017).

3. Results and discussion

3.1 Microbiological status

No *Salmonella* and *Vibrio cholerae* were found in any sample. *Escherichia coli* were found in eight samples, *L. adecarboxylata* were found in five samples, and *A. caviae* were found in eight samples (Table 3). Non-appearance of *V. cholerae* and *Salmonella spp* in all samples might be due to the fishes being preserved properly in chilled conditions. This result was similar to Thi *et al*. (2016) as they did not find any *Salmonella* and *V. cholera* in Vietnamese *Pangasius hypophthalmus* during processing. However, some research found *Salmonella* in pangas fish collected from different wholesale and retail markets in Dhaka, Bangladesh (Hasan *et al*., 2015).

Total plate count (TPC) of fish varied with their

Table 1. Primer sequence used for PCR amplification.

Primers	Sequences (5'-3')	Primer size (bp)	GC content (%)	PCR amplification size (bp)
8F	AGAGTTTGATCCTGGCTCAG	20	50.0%	1484
1492R	GGTTACCTTGTTACGACTT	19	42.1%	

Table 2. The concentration of PCR mixture.

SN.	Reagents	Concentration	Final volume (100 µL)
1.	25mM $MgCl_2$ (Thermo Fisher Scientific)	1.5 mM	6
2.	Reaction buffer (Thermo Fisher Scientific)	1×	10
3.	10mM dNTP (Thermo Fisher Scientific)	200 µM each dNTP	2
4.	F primer (Macrogen Korea)	0.1-1.0µM	3
5.	R primer (Macrogen Korea)	0.1-1.0 µM	3
6.	DNA template	100ng/100µL	5
7.	Taq polymerase (Thermo Fisher Scientific)	0.05 U	1
8.	Sterile deionized water		70

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Table 3. Antibiotic sensitivity reference table adapted from CLSI (2018).

Antibiotics	Disc content	Zone diameter (mm)		
		Susceptible (S)	Intermediate (I)	Resistant (R)
Ampicillin	10 µg	≥ 17	14-16	≤ 13
Ciprofloxacin	5 µg	≥ 21	16–20	≤ 15
Gentamicin	10 µg	≥ 15	13–14	≤ 12
Nitrofurantoin	300 µg	≥ 17	15–16	≤ 14
Levofloxacin	5 µg	≥ 17	14–16	≤ 13
Chloramphenicol	30 µg	≥ 18	13–17	≤ 12
Tetracycline	30 µg	≥ 15	12–14	≤ 11
Azithromycin	15 µg	≥ 13	-	≤ 12
Trimethoprim	5 µg	≥ 16	11–15	≤ 10

Table 4. Frequency of bacteria isolated from pangas fish of different fish market.

SN.	Bacterial Isolates	Frequency No (%)	Area		
			Chowrasta	Shimultoli	Joydebpur
1	*Salmonella*	0 (0)	-	-	-
2	*Vibrio cholerae*	0(0)	-	-	-
3	*Escherichia coli*	8(38.09)	+	+	+
4	*Leclercia adecarboxylata*	5(23.80)	+	-	+
5	*Aeromonas caviae*	8(38.09)	+	-	-
	Total	21(100)			

different source (Table 4). The highest TPC was 5.22 Log_{10} CFU/g, fish collected from Chowrasta and the lowest was 4.79 Log_{10} CFU /g, the source was Shimultoli fish market. A significant difference ($p < 0.05$) was found among the TPC of the three Log_{10} markets. Bacterial quantity in food less than 6-7 Log10 CFU/g is considered suitable for human consumption (ICMSF, 1986). The TPC in pangas was within the acceptable limit. A similar result was observed in pangas fish in the retail market in Dhaka city, where the range of TPC was 5.66 Log_{10} CFU/g to 5.85 Log_{10} CFU/g (Hasan *et al*., 2015). Relekar *et al*. (2019) stated that TPC in raw pangas meat was 5.55 Log_{10} CFU/gm in Nagpur, India. The overall bacterial load of the three fish markets in the Gazipur district has an acceptable limit. The TPC of the three fish markets was different, which might be due to the difference in bacterial load natural as the total plate count can be varied due to natural microflora carrying of aquatic organisms, feeding habits, water source, water quality, habitat structure, stocking density between culture systems, and environment (Prieur *et al*., 1990). In the present research, pangas were collected during summer (March to June) from the retail fish market. However, different environments and seasons could result in different bacterial quantities (Ray and Bhunia, 2007).

Escherichia coli was found in pangas fish collected from all markets, ranging from 2 Log_{10} CFU/g (Chowrasta fish market) to 2.698 Log_{10} CFU/g (Shimultoli fish market) (Table 4). *Escherichia coli* (38.09% of isolates) was found in eight samples only (Table 5). Although the range is within the acceptable limit, the presence of this microorganism in fish from all three sources is not a good sign. The presence of *E. coli* in pangas fish indicates unhygienic and improper handling during harvesting, storage, or sale in a fish market or fecal contamination occurred at some point (Mandal *et al*., 2009; Roy *et al*., 2013). The highest amount of *E. coli* in the Shimultoli fish market indicates that this market had more unhygienic sanitary conditions and poor handling practices than the other two markets. *Escherichia coli* is a thermotolerant coliform and natural microflora of intestinal tracts of warm-blooded animals, which cause different enteric diseases (Da Silva *et al*., 2012). The water in Bangladesh can be contaminated during the 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. In Bangladesh, *E. coli* was found in Nile tilapia collected from a fish market (Mandal *et al*., 2009). This result indicates that fish were contaminated with *E. coli*, indicative of poor sanitation practices at some point in the supply chain.

Around 38.09% of isolates were *Aeromonas caviae* found in pangas fish collected from the Chowrasta fish market (Table 5). In Bangladesh, *Aeromonas* spp. were isolated from pangas and other species (Abedin *et al*., 2020). *Aeromonas caviae* was isolated from fish hatcheries, carp farms, ornamental fish and frozen fish in India, Syria, Srilanka and Egypt, respectively (Dhanapala *et al*., 2021; Hafez *et al*., 2018; Daood, 2012; Bharathkumar and Abraham, 2011). In Vietnam and

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Malaysia, *Aeromonas* spp. was found in pangas fillet and pangas fish, respectively (Fauzi *et al*., 2021; Thi *et al*., 2016). Among 36 species, motile *Aeromonas* spp. are important fish pathogens known as motile *Aeromonas* septicemia (MAS) (Dhanapala *et al*., 2021). *Aeromonas caviae, A. hydrophila, A. sobria* and *A. veronii* are linked with MAS, causing high mortality of fish, also known as red sore, hemorrhagic septicemia or epizootic ulcerative disease (EUD) and *A. caviae, A. hydrophila, A. dhakensis* and *A. veronii* are associated with human infection (Hossain *et al*., 2019; Syrova *et al*., 2018; Parker and Shaw, 2011). So, the presence of *Aeromonas* spp. in pangas can be an important threat to public health. The genus *Aeromonas* causes intestinal and extra-intestinal disease, and gastroenteritis occurs due to the consumption of *Aeromonas* contaminated food or water (Dhanapala *et al*., 2021; Janda and Abbott, 2010). Children, older people, and immunocompromised patients face threatened conditions by *Aeromonas* infection (Janda, 1991). Most critical thing is *Aeromonas* species can grow and produce toxins in refrigerated conditions (Eley *et al*., 1993). It indicates that refrigeration cannot be enough to control this microorganism (Kirov, 1993).

The possible causes for getting this bacteria in pangas fish might be the improper handling of fish or unhygienic water conditions in which fishes were cultured. As this species is the reason for several fatal human diseases, including wound infections, gastroenteritis and septicemia, there is a need for public consciousness about the outbreak of diseases in humans through ingestion of the bacteria along with fish. The preventive method should be taken during food preparation; fish should be thoroughly cooked before consumption. Good personal hygiene and proper sanitation procedures should always be used to prevent human exposure to this pathogen. Also, the preservation times should be shortened in markets and houses as pathogens can survive and grow in refrigerated conditions.

Around 23.80% of isolates were *Leclercia adecarboxylata*, isolated from fishes of Chowrasta and Joydebpur fish market (Table 4 and Table 5). *Leclercia adecarboxylata* is a motile, aerobic, gram-negative bacillus, 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 (Spiegelhauer *et al*., 2019), its presence in fish is not unusual. It was found in traditional dried anchovies (*Encrasicholina punctifer*) in Oman and gilt-head bream (*Sparus aurata)* fish in Portugal (Bulushi *et al*., 2013; Salgueiro *et al*., 2020). This species is present in the gut of animals (Hess *et al*., 2008). 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 pangas fish or other fishes in Bangladesh has not been reported yet. Previously, *L. adecarboxylata* were isolated from various clinical specimens such as blood, feces, sputum, urine, and responsible for bacteremia, sepsis, peritonitis, cellulitis, endocarditis, and cholecystitis (Garcia-Fulgueiras *et al*., 2014). It can cause monomicrobial infection in an immunocompromised patient (Anuradha, 2014). People who already suffer from primary diseases such as cancer, leukemia, and cirrhosis can be easily affected by pneumonia, septicemia, and wound infections through this pathogen (Matsuura and Sugiyama, 2018; Haque *et al*., 2015).

As this species is already found in the river of Dhaka city (Haque *et al*., 2015), it can easily contaminate fish in different ways. The possible sources of *L. adecarboxylata* might be unhygienic handling and cross-contamination during icing, storage, and selling in the fish market, as significantly less hygiene is maintained in the fish market in Bangladesh. It is harmful, so the presence of this microorganism in any fishery products is not safe to eat. If fish are stored in cool conditions and maintained in hygienic conditions in every step from processing to marketing, in that case, it might be possible to minimize the possibility of the presence of this harmful bacteria.

3.2 Antibiotic sensitivity

Aeromonas caviae showed the most sensitivity against ciprofloxacin and resistance against ampicillin (Table 6, Figure 1). Both sensitive and intermediate responses were observed towards gentamycin (37.5% S and 62.5% I), nitrofurantoin (25% S and 75% I), levofloxacin (37.5% S and 62.5% I), chloramphenicol (50% S and 50% I) and oxytetracycline (62.5% S and 25% I) (Table 6). No sensitivity was observed against

Table 5. Different type of bacteria isolated from pangas fish.

SN	Location	TPC (Log_{10} CFU/g)		*E. coli* (Log_{10}CFU /g)	
		Minimum	Maximum	Minimum	Maximum
1	Chowrasta	4.946	5.220	2.000	2.602
2	Shimultoli	4.795	5.035	2.544	2.698
3	Joydebpur	4.903	5.058	2.217	2.267

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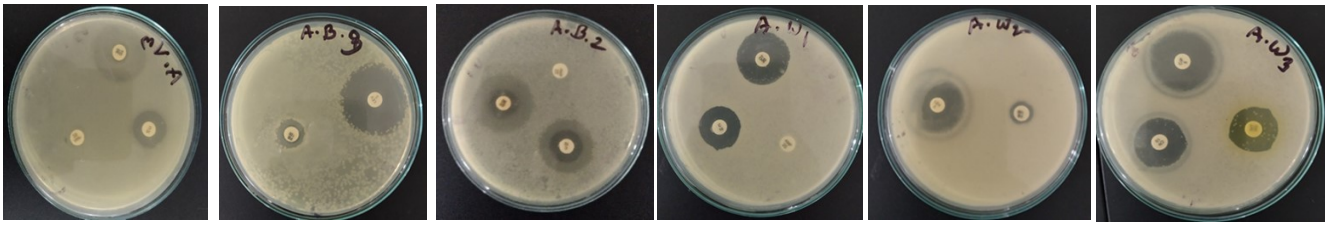
Table 6. Antibiotic susceptibility pattern of *A. caviae.*

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin (10 µg)	0	0	0	0	8	100
Ciprofloxacin (5µg)	8	100	0	0	0	0
Gentamycin (10 µg)	3	37.5	5	62.5	0	0
Nitrofurantoin (300 µg)	2	25	6	75	0	0
Levofloxacin (5 µg)	3	37.5	5	62.5	0	0
Chloramphenicol (30 µg)	4	50	4	50	0	0
Oxytetracycline (30 µg)	5	62.5	2	25	1	12.5



a b c d e f

Figure 1. Antibiotic resistance pattern of *Aeromonas caviae* (a, b), *L. adecarboxylata* (c, d) and *E. coli* (e, f) isolates.

ampicillin, and only one isolate showed resistance to oxytetracycline (12.5%). However, no resistance was observed against ciprofloxacin, gentamycin, nitrofurantoin, levofloxacin and chloramphenicol. Additionally, no intermediate response was observed against ampicillin and ciprofloxacin (Table 6). These findings somehow agreed with the resistance profile report of *Aeromonas* spp. in Bangladesh, Egypt, Syria, Nigeria and India (Abedin *et al*., 2021; Hafez *et al*., 2018; Daood, 2012; Ashiru *et al*., 2011; Thayumanavan *et al*., 2003). Their report mostly showed resistance to ampicillin and tetracycline and sensitivity to ciprofloxacin. In this study, *A. caviae* showed 100% sensitivity to ciprofloxacin and 100% resistance to ampicillin. Similarly, Kaskhedikar *et al*. (2010) found *Aeromonas* sp. was 100% susceptible to ciprofloxacin, and all the isolates were resistant to ampicillin. Although this research found that *A. caviae* reaction towards chloramphenicol and gentamycin was both sensitive and intermediate, some reports found *Aeromonas* sp. resistance to chloramphenicol, gentamycin and ciprofloxacin (Bharathkumar and Abraham, 2011; Daood, 2012; Hafez *et al*., 2018).

Leclercia adecarboxylata showed the most sensitivity to ciprofloxacin (100%) and the most resistance to ampicillin (Table 7). Both sensitive and intermediate responses were observed towards gentamycin, nitrofurantoin, levofloxacin, chloramphenicol and oxytetracycline (Table 7). No sensitivity was found against ampicillin, while *L. adecarboxylata* did not show any resistance against ciprofloxacin, gentamycin, nitrofurantoin, levofloxacin, chloramphenicol and oxytetracycline (Figure 1). The present research somehow supports the previous report 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; Anuradha, 2014). 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* were 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). Although present research used only seven antibiotics, results were consistent for common antibiotics except ampicillin, all isolates showed sensitivity to all antibiotics.

Escherichia coli also showed the most resistance to ampicillin (Table 8). Both sensitive and intermediate responses were observed towards ciprofloxacin (87.5 % S and 12.5% I), gentamycin (50 % S and 50% I), nitrofurantoin (50% S and 37.5% I), levofloxacin (62.5 % S and 37.5 % I), chloramphenicol (50 % S and 50% I) and oxytetracycline (37.5 % S and 37.5% I) (Table 8). No sensitivity was found against ampicillin, whereas *E. coli* did not show any resistance against ciprofloxacin, gentamycin, levofloxacin and chloramphenicol (Figure 1). Interestingly, *E coli* showed resistance to oxytetracycline (25%) and nitrofurantoin (12.5%). The previous report of resistance of *E coli* isolated from seafood is similar for ampicillin and tetracycline; however, the report also shows resistance toward chloramphenicol, ciprofloxacin and gentamycin (Ryu *et al*., 2012). *Escherichia coli* isolated from stool samples shows resistance mostly to ampicillin, amoxicillin and

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Table 7. Antibiotic susceptibility pattern of *L. adecarboxylata.*

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin (10 µg)	0	0	0	0	5	100
Ciprofloxacin (5µg)	5	100	0	0	0	0
Gentamycin (10 µg)	4	80	1	20	0	0
Nitrofurantoin (300 µg)	3	60	2	40	0	0
Levofloxacin (5 µg)	4	80	1	20	0	0
Chloramphenicol (30 µg)	2	40	3	60	0	0
Oxytetracycline (30 µg)	3	60	2	40	0	0

Table 8. Antibiotic susceptibility pattern of *E. coli* isolates.

Antimicrobial Agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin (10 µg)	0	0	0	0	8	100
Ciprofloxacin (5µg)	7	87.5	1	12.5	0	0
Gentamycin (10 µg)	4	50	4	50	0	0
Nitrofurantoin (300 µg)	4	50	3	37.5	1	12.5
Levofloxacin (5 µg)	5	62.5	3	37.5	0	0
Chloramphenicol (30 µg)	4	50	4	50	0	0
Oxytetracycline (30 µg)	3	37.5	3	37.5	2	25

ciprofloxacin (Erb *et al*., 2007).

Although ampicillin is used to treat infections of the respiratory tract, skin and urinary tract (Duran and Marshall, 2005), no isolates showed sensitivity to ampicillin. Resistance towards ampicillin might be due to the overuse of this drug in livestock and fish farming and, as a result, the adaptation of bacterial quantity with ampicillin. In this study, most of the bacteria were susceptible to ciprofloxacin. However, one isolate of *E. coli* was in the intermediate range. Ciprofloxacin is the metabolite of enrofloxacin and is used in firming to control gram-negative bacteria and, in some cases control gram-positive bacteria (Bermúdez-Almada and Espinosa-Plascencia, 2012).

Similarly, all isolates were sensitive and intermediate for gentamycin and levofloxacin. For *Proteus* infection, farmers used gentamycin and *Aeromonas* infection, used quinolone or levofloxacin (Cao *et al*., 2007; Jones and Wilcox, 1995). So, sensitivity for gentamicin and levofloxacin is a good result for disease control.

Interestingly, banned antimicrobial drugs (nitrofurantoin, chloramphenicol) showed sensitive characteristics for all isolates except one for *E. coli* (nitrofurantoin 12.5%). Nitrofurantoin and chloramphenicol are banned for use because they are carcinogenic agents, but they still might be used in fish farming illegally. Several consignments of Bangladesh shrimp were rejected by different countries for the presence of nitrofuran drugs (Shamsuzzaman and Biswas, 2012).

In this study, oxytetracycline was also very effective against most isolates, and only two isolates (*A. caviae* and *E. coli*) showed resistance to oxytetracycline (Table 6 and Table 8). It indicates that there may be indiscriminate use of this specific antibiotic causing resistance. Oxytetracycline is the most widely used antibiotic in shrimp farming, which may be due to its effectiveness. The incidence of resistance to oxytetracycline is increasing (Duran and Marshall, 2005). As oxytetracycline is used widely in fish farming, bacteria isolates in the intermediate and resistance zone is not surprising. FDA banned oxytetracycline used as a growth promotor (Granados-Chinchilla and Rodríguez, 2017).

Disease outbreaks in fish farming are very common in Bangladesh (Faruque *et al*., 2008). Antibiotics are used to prevent and treat diseases, but unregulated and indiscriminate uses are observed in many parts of the world, especially in Asia and Africa. Faruque *et al*. (2008) reported that most farmers could not recognize the actual disease. Failure to recognize the disease is another cause for the development of antibiotic resistance as inappropriate antibiotics are used. This study indicates that antibiotic-resistant bacteria are present in retail fish. In the future, routine inspection is needed for the occurrences and antibiotic resistance pattern of *A. caviae, E. coli* and *L. adecarboxylata* isolated from fish and other aquatic environments. Additionally, training for stakeholders in fisheries is essential to avoid the emergence of multi-drug resistant bacteria.

Based on the research findings, there are several suggestions to improve food safety and quality for consumers. From selling to transportation from the farm to different markets around Bangladesh, unwanted

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bacteria have a high chance of cross-contamination. Therefore, good aquaculture practices should be applied at the farm level regardless of the final destination, including good water quality, regular water exchange, proper handling during harvesting, transportation and storage. After purchase, people should adequately wash and cook all seafood. Good aquaculture practices, proper handling and awareness education among farmers and consumers can decrease the chance of contamination by harmful bacteria. It needs to create a policy and plan for solutions to the problems of pathogenic bacteria in the retail fish market and their resistance. This policy should have specific goals that maintain proper hygiene in every step, from fishing to distribution to consumers and finding alternative antibiotics such as probiotics. An appropriate timeframe will be needed, and within that time, monitoring should be applied for permitted antibiotics with proper doses and a prescribed system for selling antimicrobial drugs. This study provides some important information about the quality and safety of retail market pangas fish, which will be helpful for researchers and consumers all over the world.

4. Conclusion

Studies were conducted to investigate the total plate count (TPC), the presence of morbific bacteria in pangas fish, and the antibiotic susceptibility of isolated bacteria. No *Salmonella* and *V. cholerae* were found, however, *E. coli*, *L. adecarboxylata* (accession no. MN625850, MN625851) and *A. caviae* (accession no.MN625853) were found. Seven antibiotics were used for antibiotic resistance testing, and isolated bacteria mostly showed resistance toward ampicillin, and only two isolates showed resistance to nitrofurantoin and oxytetracycline. The highest sensitivity showed for ciprofloxacin. Some bacteria are natural microflora in pangas farms, but the presence of *E. coli* indicated that fecal contamination occurred at some point in the supply chain. This study indicates that pathogenic bacteria present in retail pangas fish of Gazipur district. However, it is critical that all fish be handled correctly from harvest to consumption and that proper hygiene is practiced at all levels. Regulations about good aquaculture practice are already present in Bangladesh, but they are challenging to implement and inspect. It should be started at the farm in the form of clean surroundings, water from good sources, good sanitary conditions and awareness development of farmers about antibiotic use through proper training. Proper laws and policies need to be enforced and implemented to ensure food safety related to fish. The presence of those bacteria and their antibiotic resistance indicates that 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.

Conflict of interest

The authors declare no conflict of interest.

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