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Research Article

Prevalence and distribution of antimicrobial resistance profile of *Escherichia coli* isolated from various local fish markets in Dhaka city, Bangladesh

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ABSTRACT

Considering the importance of the fish and fish products as a vital part of the human diet in Bangladesh, the study aimed to estimate the microbial load, identify the *E. coli* and observe the isolates antibiogram pattern. Standard plate count technique, as well as the 3-tube most probable number (MPN) method, were applied to estimate the total viable bacteria (TVB) and total coliforms (TC), total fecal coliforms (TFC), and *E. coli*, respectively. Approximately 61.91% of samples fell into the marginally acceptable limit for TVB count, while 66.67% and 95.25% of examined species exceeded the threshold limit for TC and TFC, respectively ($p<0.05$). Around 90% of samples were contaminated with *E. coli*. A culture sensitivity test revealed that cent percent strains harbored resistance to penicillin, rifampicin, and erythromycin. Multiple antibiotic resistance (MAR) index above 0.2 may indicate the misuse or overuse of antibiotics. The findings highlight the potential food safety hazards associated with fish concerning the random use of antibiotics.

Introduction

Fish and all the fish items are the primary sources of their protein, and there is a growing recognition of their nutritional and health-promoting qualities. The products of fish are also considered a major source of earning and livelihood for numerous communities worldwide (Nowsad et al., 2015). Bangladesh is recognized as the world's foremost fish-producing country, where people also depend on fishing, fish farming, processing, and trading for their earnings (DoF, 2018). Comparatively, Bangladeshi people eat more fish as a source of animal protein which covers 60% of their demand, than meat products (DoF, 2018). From CDC (USA) data, it has been known that 3% people of United States acquired foodborne illnesses from contaminated fish (Barrett et al., 2017). So, consuming more fish may be a great concern for food security. FAO reported that each year nearly 30 million people are affected by food-borne diseases in Bangladesh (FAO, 2012). Though the involvement of fish causing food-borne illness is not clear in this tropic, several microorganisms that are normal inhabitants in the aquatic environment may cause contamination (Dutta et al., 2018).

Due to the dense population and poor sanitation facilities, the people of Bangladesh are more susceptible to microbial attacks and face the challenges of fecal contamination (Singh et al., 2020). A large group of the population involved in unhygienic sanitary practices mostly live beside the haor, baor, beel, pond, and river. Open defecation and inadequately treated or untreated domestic sewage disposal may contaminate these natural water bodies with several human pathogenic microbes (Dutta, 2016). In most cases, domestic local markets' surroundings remain soggy, filthy, and unhealthy

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(Begum et al., 2015). Unhygienic landing sites, poor handling, transport, storage, display, and packaging process may increase the risk of microbial contamination of fish (Costa, 2013).

Consequently, consumers may develop infection or intoxication (Assefa et al., 2019). Besides food safety issues, intake of contaminated fish may increase the risk of developing antimicrobial resistance (Ng et al., 2018). The extensive, random, and misuse of antibiotics for therapeutic purposes or as growth promoters in livestock or as feed additives in fish farms caused a genetic selection of more harmful bacteria, a matter of great public health concern (Founou et al., 2016). A member of the coliform group, *Escherichia coli* is found in the intestinal tract of the animals, humans, and fish microbiota (Cardozo et al., 2018). It is a widely accepted fecal contamination indicator organism of fish and water (Price and Wildeboer, 2017). It can be easily propagated in various living ecosystems and interchange genetic material with other bacterial communities that may lead to the emergence of resistant bacteria, ultimately causing the disease in humans (Ryu et al., 2012). A higher frequency of resistant *E. coli* strains has been noticed in fish and seafood in India (Kumar et al., 2005) and Korea (Ryu et al., 2012), indicating the urgency of monitoring the presence of the bacteria in our region (Boss et al., 2016). Limited studies were performed in our country regarding this point. The specific objectives of the study were: 1) to get insight into the microbial status in fresh fish samples 2) identification of the major fecal coliform *E. coli*, and 3) to determine the antibiogram profiles of *E. coli*, and observe their multidrug-resistant nature.

Materials and Methods

Field sampling

Between March and August 2019, a total of 21 fish samples were purchased from three different local fish markets (Karwan bazar, Khilgaon bazar, and Shyambazar) located at three zones in the city of Dhaka, Bangladesh (Fig. 1). The samples included in this study were the following seven different categories: Koi (*Anabas testudineus*), Poa (*Otolithoides pama*), Loitta (*Harpadon nehereus*), Sorputi (*Puntius sarana*), Rupchanda (*Pampus chinensis*), Bhata (*Labeo bata*) and Taki (*Channa punctate*). Each type of fish sample was aseptically collected after a one-week interval into sterile plastic boxes, afterward placed in a cool box, and immediately transported to the laboratory. The samples were processed as early as possible for microbiological analysis, i.e., total viable bacterial count (TVBC), total coliform count (TCC), and total fecal coliform count (TFCC).

Sample processing and media preparation

About 20 g of the muscle of individual fish samples were weighed aseptically in an analytical balance (Shimadzu, Japan) in a triplicate fashion. After that, samples were crushed for 2 minutes in a pre-sterile blender containing 180 mL of sterilized normal saline at room temperature to make proper homogenization. Various nutrient broths and agar used in this study were nutrient agar, lauryl tryptose broth (LTB), 2% brilliant green bile broth (BGBB) and MacConkey broth. All of these media were prepared according to Cheesbrough (1984) and autoclaved at 121°C for 15 min before use.

Quantitative analysis of total viable bacteria

The aerobic plate count (APC) method enumerated the total microbial load (FAO, 1992). Nutrient agar was used in this method. Each sample was serially diluted up to 10^{-4} dilutions with 9 mL of sterile 0.85% NaCl solution. An aliquot of 0.1 mL diluted fish sample was placed on the agar plates (Hi-Media, India) using a spread plate technique. Plates were incubated at 37 °C for 18-24 hours, followed by cell counting.

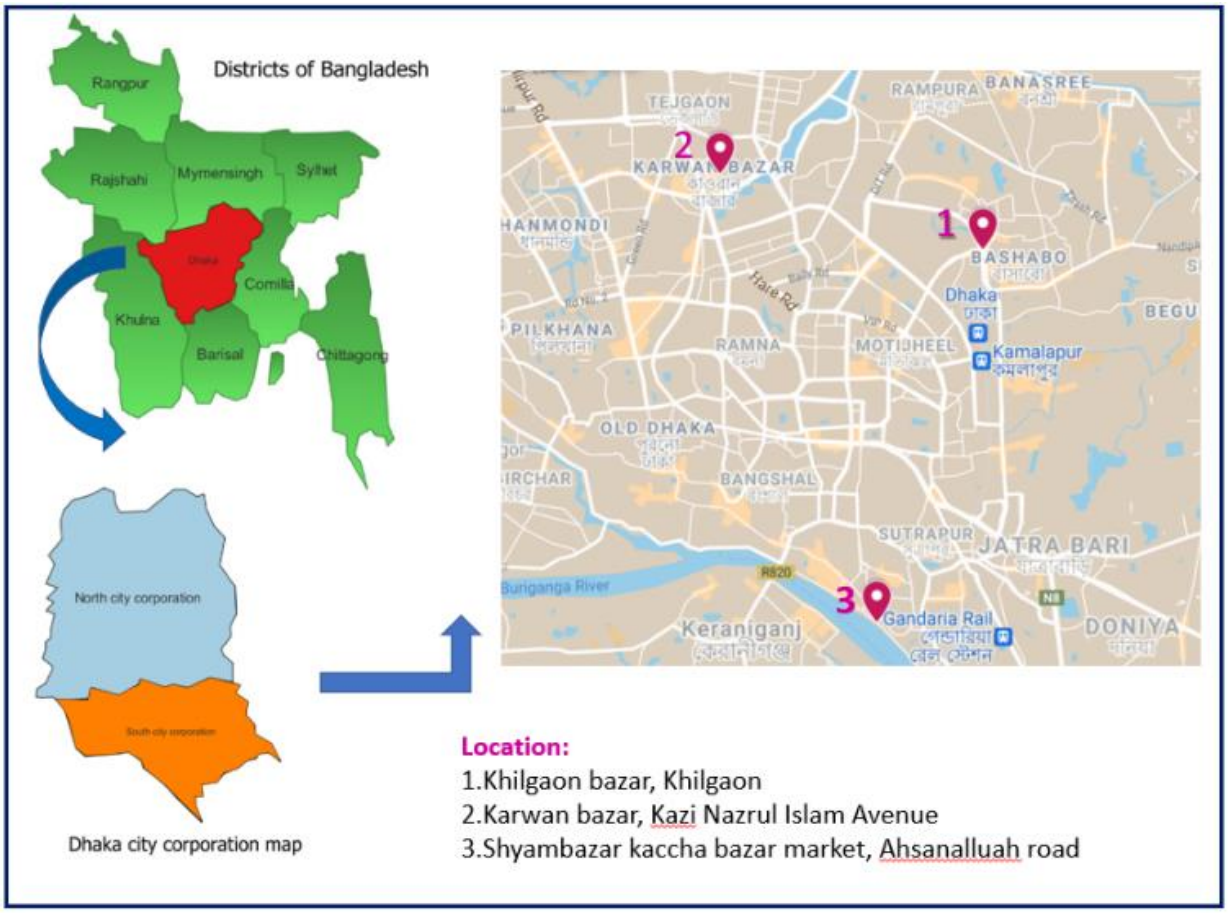

Fig. 1. The map of Dhaka city indicates the area of sampling sites

Estimation of total coliforms and fecal coliforms

Lauryl tryptose broth (LTB), 2% brilliant green bile broth (BGBB), and MacConkey broth were used to estimate the number of total coliforms and fecal coliforms. According to standard protocol, the most probable number (MPN), the most conventional 3-tube method, was employed here (FAO, 1992). A similar decimal serial dilution series for each sample was performed here (i.e., 9 mL LTB plus one 1 mL crushed fish sample). A triplicate set of three test tubes filled with 9 ml LTB (Hi-Media, India) were sterilized. A one ml of ten-fold serial dilutions (up to 10^{-3}) were inoculated into triplicate sets, respectively and incubated at 37 ^{0}C for 24-48 hours. Gas and acid produced from lactose fermentation point out the presumptive heterofermentative metabolism of coliforms (FAO, 1992). To confirm the presence of total coliforms and fecal coliforms bacteria, approximately one-loopful inoculum of each gas positive LTBs were placed in the pre-sterilized test tube containing BGBB (Oxoid, UK) at 37 ^{0}C for 48 hours and MacConkey broth (Oxoid, Uk) at 44.5±0.5 ^{0}C for 48 hours respectively. After incubation, the gas produced in the tubes was recorded and compared with the MPN chart to count the total coliforms and total fecal coliforms number.

Isolation and Identification of E. coli

From each gas positive MacConkey broth, one loopful fecal coliforms sample was streaked on an eosin methylene blue (EMB) agar (Scharlau, Spain) plate. The plates were incubated at 37 ^{0}C and examined after 24-48 hours for bacterial growth and any change of color in the media. Black colonies with a metallic green sheen were observed on the EMB agar plate, indicating the presence of *E. coli*. After Gram's staining of suspected bacterial

colonies, several biochemical tests such as indole production, methyl-red, Voges-Proskauer and citrate utilization (IMViC) tests were performed according to standard microbiological guidelines for the identification of *E. coli* (FAO, 1992).

Antimicrobial susceptibility testing

The bacterial isolates were subjected to a culture sensitivity test on a Mueller-Hinton agar (MHA) (Hi-Media, India) plate according to the Kirby-Bauer disc diffusion method (Bauer et al., 1966). A total of twelve panels of commercial antibiotic disks (Oxoid, UK) were used at different concentrations. The discs strength were: Cefixime, Rifampicin and Ciprofloxacin at 5 µg/mL; Gentamicin, Ampicillin, Penicillin, Streptomycin and Norfloxacin at 10 µg/mL; Erythromycin at 15 µg/mL and Tetracycline, Chloramphenicol and Neomycin at 30 µg/mL. A suspension of *E. coli* isolates was prepared equivalent to 0.5 McFarland standards and was made as lawn cultures onto sterile Mueller Hinton agar plates. The antibiotic discs were placed on the surface of the plates using sterile forceps and were incubated at 37 ^{0}C for 18-24 hours. Criteria for the sensitivity, intermediate, and resistance were recorded as per Clinical Laboratory Standards Institute (CLSl) guidelines, 2012. Multidrug-resistant (MDR) *E. coli* was defined according to the international expert proposal for interim standard definitions for acquired resistance. Further multiple antibiotic resistance (MAR) index of only multiple drug-resistant (MDR) *E. coli* isolates was calculated. Krumperman (1983) represents the MAR index, calculated by the ratio of the digit of antibiotics to which the isolate was resistant (a) and the digit of antibiotics to which the isolate was subjected (b).

Statistical analysis

All the data were analyzed and filtered in Microsoft Excel version 2012. The APC data were converted to $\log_{10}$ formed for ease of calculation. Descriptive statistics (graphs) were applied to visualize the outcome, and a p-value ≤ 0.05 was considered as significant for mean values.

Results and Discussion

All samples' average total viable bacterial count (TVBC) was 6.30 $\log_{10}$ cfu/g, ranging from 4.08-6.47 $\log_{10}$ cfu/g. The mean value of the total viable bacterial count belonging to seven different fish species was varied (Fig. 2). The highest mean of TVBC was found in Rupchanda (6.57±0.66 $\log_{10}$ cfu/g), whereas the lowest mean count (4.80±0.62 $\log_{10}$ cfu/g) was observed in the Taki.

The current study reported the microbiological quality of the fishes following the ICMSF, 1986 criteria into three categories: good (>5×10^5 cfu/g), marginally acceptable (5×10^5 cfu/g to 10^7), and unacceptable (<10^7). Based on the criteria, 100% of samples of Taki were good microbiological limits. In contrast, cent percent of Loitta and Poa fishes and 66.67% of Rupchanda fishes were marginally acceptable quality (Fig. 3). Notably, 33.33% of Rupchanda fishes with unacceptable limits were noticed. Overall, the TVB count of the 33.33% samples was within the range of good quality, whereas 61.91% and 4.76% samples fell into marginally acceptable ($p<0.05$) and unacceptable limits, respectively. Hence, statistically significant percentages of the samples, 66.67%, were beyond the good quality limit ($p<0.05$).

Several previous investigations performed on various fishes from retail shops also reflect the same scenario. The TVBC ranges from 6×10^4 to 1.6×10^6 cfu/g (Sarkar et al., 2020), 2.89×10^5 to 2.98×10^7 cfu/g (Begum et al., 2015), and 3.3×10^5 cfu/g to 1.9×10^8 cfu/g (Begum et al., 2010) comply with the outcomes of the present study. So, the fish samples of domestic markets contain a high bacterial load that may be transmitted to the consumers and the people associated with the transportation, handling, and processing through contact (Ribeiro et al., 2016). The outcomes provide an initial alarm to safeguard fish consumers from the chance of infection and demand an urgent step to uplift the quality control systems of the local shops.

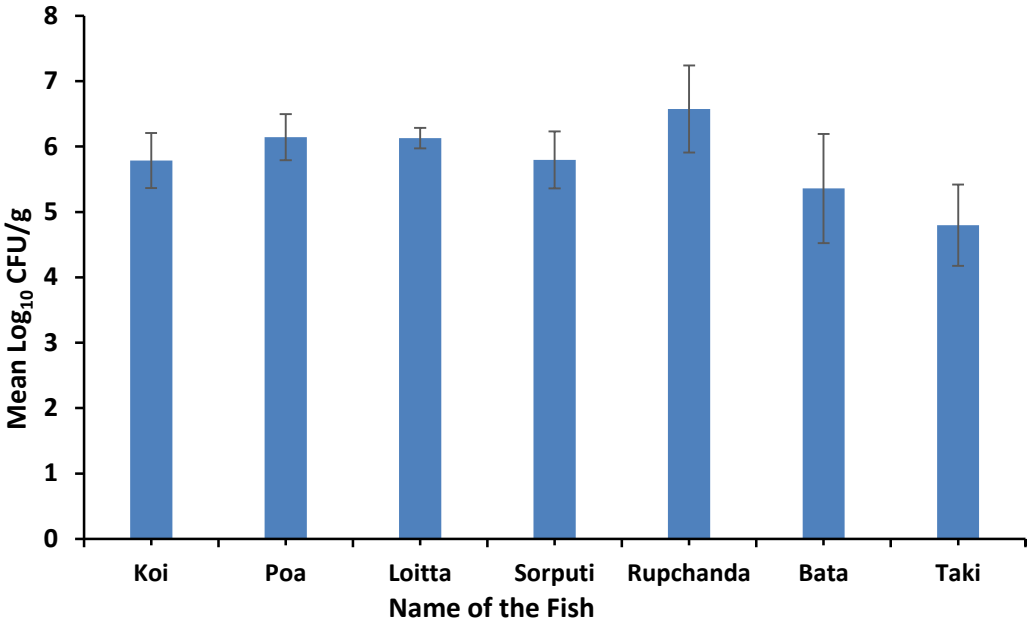

Fig. 2. Total viable bacterial count (log_{10} cfu/g) of the raw fish samples collected from local markets. The values were represented with mean ± SD.

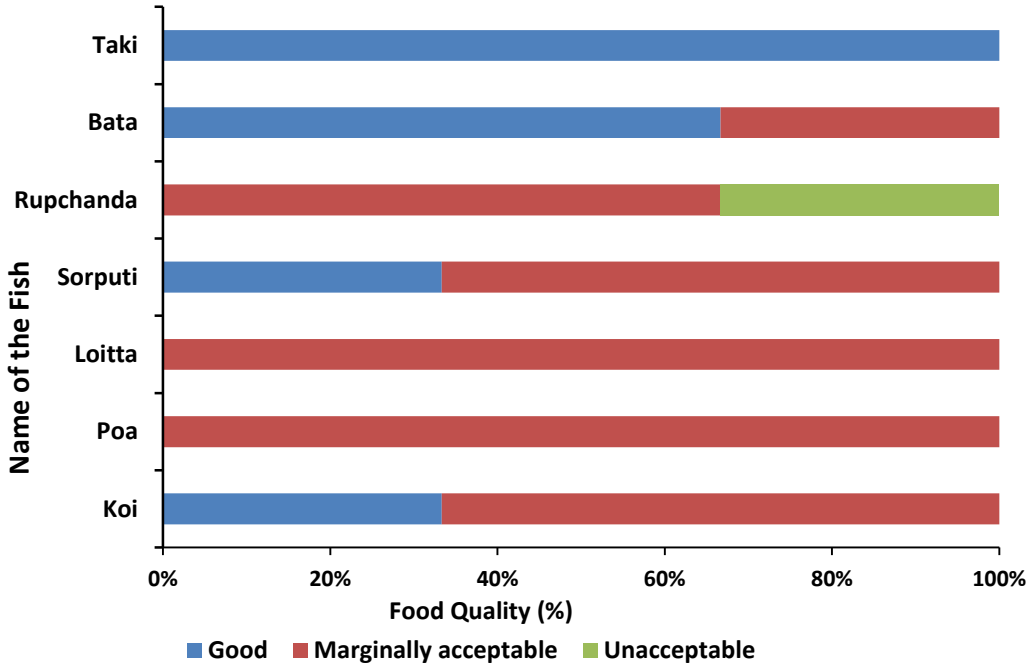

Fig. 3. Microbiological quality of the fishes according to the International Commission on Microbiological Specifications for Food (ICMSF).

Nearly all the representative fish samples were contaminated with coliforms and fecal coliforms bacteria. The bacteriological qualities of the fish samples brought from the local markets are summarized in Table 1. In the present research work, the total coliforms count (TCC) ranged between 2.1 and >1100 MPN/g, and the total fecal coliforms count (TFCC) was in the range of 3.6 MPN/g to >1100 MPN/g. The maximum TCC and TFCC count was found on Koi and Poa fish (>1100 MPN/g) from Shyambazar and the Taki fish from Khilgaon Bazar. The lowest TCC count was present in Loitta fish (2.1 MPN/g) of Shyambazar, while the Taki fish (3.6 MPN/g) of Karwan bazar exhibited the least TFCC count. ICMSF establishes the standard acceptance limits of total coliforms (TCC) and fecal coliforms (TFCC) for fresh fish that are <100 MPN/g and <10 MPN/g, respectively. The present study found that almost 66.67% and 95.25% of examined species exceeded the acceptance limit significantly ($p<0.05$) for coliforms, and fecal coliforms, respectively.

Table 1. The total coliform (TC) and total faecal coliform (TFC) count assessment of raw fish samples.

Sampling Sites	Microbial Load (MPN/g)	Name of the Fish						
		Koi	Poa	Loitta	Sorputi	Rupchanda	Bata	Taki
LM-1	TCC	>1100	>1100	2.1	35	210	1100	210
	TFCC	>1100	>1100	210	20	28	150	28
LM-2	TCC	210	150	21	210	210	21	>1100
	TFCC	460	210	28	28	1100	23	3.6
LM-3	TCC	28	28	150	43	240	460	>1100
	TFCC	28	460	240	93	240	150	>1100

LM-1= Shyambazar, LM-2= Karwan bazar, LM-3=Khilgaon bazar, TCC= Total Coliform Count, TFCC=Total Faecal Coliform Count. MPN=Most Probable Number.

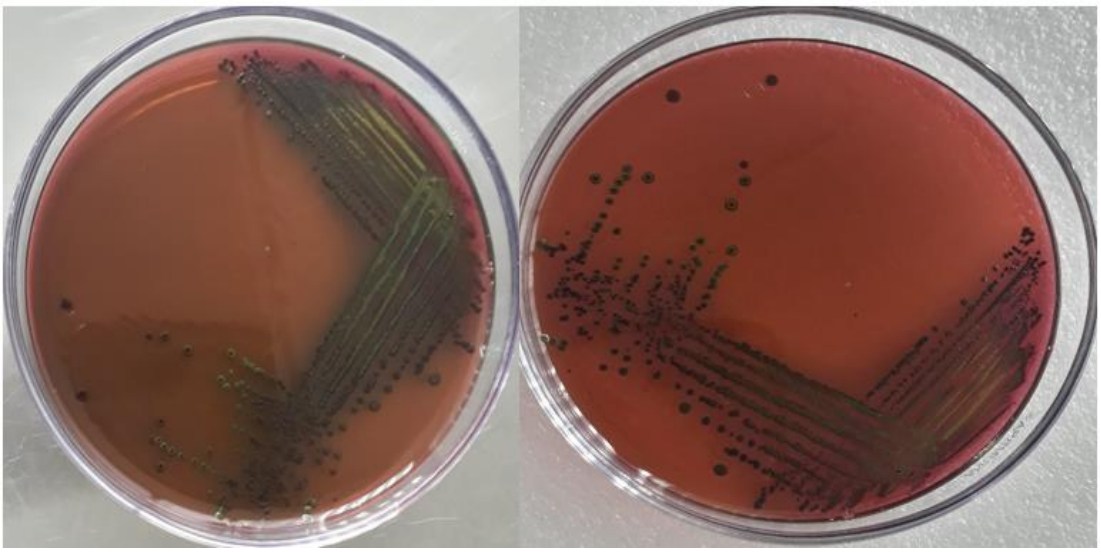

Fig. 4. Growth of *E. coli* colonies on EMB agar plate

Following the conventional culture technique, 19 out of 21 samples displayed black colonies with a metallic green sheen on the EMB agar plate (Fig. 4). All the gram-negative samples that were subjected to IMViC tests also confirmed the identification of *E. coli* strains (Table 2). In the present research finding, the overall prevalence of *E. coli* was more than 90%. The presence of the organism in the fish was 100% in the two sampling sites Shyambazar and Karwan bazar. Moreover, a lower contamination rate was reported in the Khilgaon bazar, which was 71.4%. The percentages may indicate the probability of the presence of these bacteria in higher density in freshwater. High *E. coli* load like 80.70%, 46.6% and 80% was also found in India (Dutta, 2016), Kenya (Onyuka et al. 2011), and Switzerland (Boss et al., 2016), respectively. The prevalence of coliforms and fecal coliforms at higher ranges may indicate sewage contamination. Untreated sewage is already identified as one of the principal reasons for the deterioration of water quality in the capital city of Bangladesh (Bashar and Fung, 2020).

Table 2. The results of biochemical characterization of *E. coli.*

E. coli isolates	Indole production	Methyl red	Voges-Proskauer	Citrate utilization test
19	+	+	-	-

Where + = positive and - = negative

The antibiotic disk diffusion method examined the antimicrobial sensitivity pattern of *E. coli* isolates. Following the test result, *E. coli* isolated from fish samples were entirely (100%) resistant to rifampicin, penicillin and erythromycin. In contrast, gentamycin and norfloxacin effectively suppress the growth of 94.74% of the isolates. More than 80% sensitivity was also observed in chloramphenicol and ciprofloxacin. The susceptibility percentage of candidate isolates was displayed in Fig. 5.

The antibiotic susceptibility tests revealed that all *E. coli* strains were multidrug-resistant (MDR). Isolates in this investigation were resistant to at least 3 antibiotics and at most 9 antibiotics (Table 3). The antibiogram patterns of the strains of *E. coli* are displayed in Table 3.

This observation was nearly concordant with the investigations of Pinu et al. (2007), and Ahmed et al. (2013), where strains of *E. coli* harvested from different

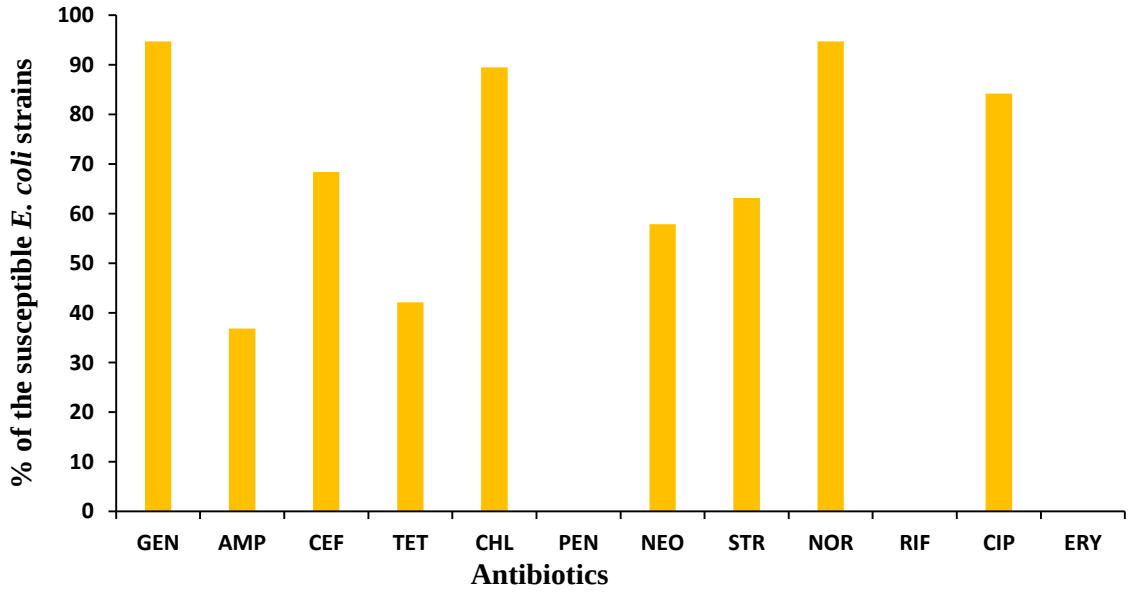

Fig. 5. The percentages of susceptible *E. coli* isolates to multiple antibiotics. The isolated strains were highly susceptible to GEN and NOR while resistant to PEN, RIF and ERY. Here, GEN= Gentamicin, Ampicillin, AMP= CEF= Cefixime, TET= Tetracycline, CHL= Chloramphenicol, PEN= Penicillin, NEO= Neomycin, STR= Streptomycin, NOR= Norfloxacin, RIF= Rifampicin, CIP= Ciprofloxacin, ERY= Erythromycin.

Table 3. The pattern of *E. coli* isolates harboring multiple antibiotic resistance

Sample No.	Source	Drug resistance patterns
1, 2, 8,12	Koi (LM-2), Poa (LM-2), Koi (LM-3), Rupchanda (LM-3)	PEN, RIF, ERY
9	Poa (LM-3)	TET, PEN, RIF, ERY
21	Taki (LM-1)	AMP PEN, RIF, ERY
5, 19	Rupchanda (LM-2) (LM-1)	AMP, TET, PEN, RIF, ERY
7	Taki (LM-2)	AMP, CEF, PEN, RIF, ERY
20	Bhata (LM-1)	TET, PEN, STR, RIF, ERY
3	Loitta (LM-2)	AMP, TET, PEN, STR, RIF, ERY
6, 10	Bhata (LM-2), Loitta (LM-3)	AMP, CEF, TET, PEN, RIF, ERY
11	Sorputi (LM-3)	AMP, TET, PEN, RIF, CIP, ERY
13	Bhata (LM-3)	CEF, NOR, CIP, PEN, RIF, ERY
14	Taki (LM-3)	GEN, AMP, NEO, PEN, RIF, ERY
4	Sorputi (LM-2)	AMP, CEF, TET, PEN, NEO, RIF, ERY
18	Sorputi (LM-1)	AMP, TET, PEN, NEO, RIF, ERY, CHL, CIP
17	Loitta (LM-1)	AMP, CEF, TET, PEN, NEO, RIF, ERY, CHL, STR

LM-1= Shyambazar, LM-2= Karwan bazar, LM-3=Khilgaon bazar. GEN= Gentamicin, AMP= Ampicillin, CEF= Cefixime, TET= Tetracycline, CHL= Chloramphenicol, PEN= Penicillin, NEO= Neomycin, STR= Streptomycin, NOR= Norfloxacin, RIF= Rifampicin, CIP= Ciprofloxacin, ERY= Erythromycin.

fish species exhibited resistance to penicillin G and erythromycin, respectively. The origins of these drug-resistant *E. coli* isolates might come from the hospital or veterinary sources. However, the data concerning the prevalence of antibiotic-resistant *E. coli* strains either in clinical or environmental sources is unavailable in Bangladesh. Moreover, antibiotics are indiscriminately used in agriculture and the animal industry in our country. Adeleke and Omafuvbe (2011) calculated a MAR index value that clarifies the potential health risk among the population and whether the distribution of resistant bacteria in a particular population is high or low. Table 4 depicts the summary of the MAR index value, which varied within the range of 0.25 to 0.75. Maximum 31.58% of strains exhibited resistance to six different antibiotics, and 5.26% showed resistance to seven, eight or nine different antibiotics. All of the *E. coli* isolates scores with MAR index above 0.2

Table 4. Multiple Antibiotic Resistance Index (MAR Index) of resistant *E. coli* isolates

No. of isolates	% of isolates	No. of the antibiotics to which the isolates were resistant	MAR Index
4	21.05	3	0.25
2	10.53	4	0.33
4	21.05	5	0.42
6	31.58	6	0.50
1	5.26	7	0.58
1	5.26	8	0.67
1	5.26	9	0.75

indicate resistance to six different antibiotics, and 5.26% showed resistance to seven, eight, or nine different antibiotics. All of the *E. coli* isolate scores with a MAR index above 0.2 indicate that the strain of a particular bacteria evolved from such an environment where multiple antibiotics are frequently used. Somehow there is a misuse or overuse of antibiotics in fish farms and related water bodies. It is hard to predict the source and routes of contamination until extensive surveillance is conducted to determine the presence and dissemination of antibiotic-resistant *E. coli.* The involvement of raw or frozen fishes in the epidemiological pathways for foodborne disease transmission has already been reported (Fleming et al., 2018). Foodborne illness related to fresh fish consumption has not yet been traced in our region, or data on this issue is still lacking. In this respect, the bacteriological assessment of local fishes appears to be the foremost issue. This kind of study generates scientific facts that would help the authority to take proper steps to maintain the microbial quality of the fishes and the hygiene of fish markets and aquatic environments.

Conclusion

In conclusion, this comprehensive study reported the poor hygiene status of our domestic markets and the pitiable quality of fish by examining the microbial levels of candidate fishes that are higher than acceptable limits. The outcomes of the research highlight the potential food safety hazards associated with fish as well as also concern about the random use of antibiotics. Since there is a limitation of sample size, the study warrants the attention of authorities for further large-scale studies to continuously monitor the microbiological quality and safety of the fishes. Besides, finding out the source of contamination and routine examination of other foodborne pathogens related to fish shall focus on future research.

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