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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 3-tube MPN method was applied to estimate the total viable bacteria (TVB) and total coliforms (TC), total fecal coliforms (TFC) and *E. coli* respectively. Of the 21 samples, the average TVB count was 6.30 $\log_{10}$ CFU/g. The TC count and TFC count were in the range of 2.1 to >1100 MPN/g and 3.6 MPN/g to >1100 MPN/g respectively. Approximately, 61.91% (13/21) samples fell into marginally acceptable limit for TVB count while 66.67% (14 of 21) and 95.25% (20/21) examined species exceeded the threshold limit for TC and TFC count respectively ($P<0.05$). Around 90% samples were contaminated with *E. coli*, confirmed by IMViC test. Culture sensitivity test revealed that cent percent strains harbored resistant to penicillin, rifampicin and erythromycin. All of the *E. coli* isolates exhibited 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 about the random use of antibiotics.

Key words: TVBC · TCC · TFCC · *E. coli* · Antibiotic Susceptibility Test · MAR Index

INTRODUCTION

Fish and all the fish items are the primary sources of protein and essential nutrients and there is a growing recognition of its nutritional as well as health-promoting qualities. The products of fish are also considered as a major source of earning and livelihoods for numerous communities around the world [1]. Bangladesh is recognized as world's foremost fish producing countries where people also earn their livelihood and income on fishing, fish farming, processing and trading [2]. Comparatively Bangladeshi people depend more on fish as a source of animal protein which cover 60% of their demand than meat products [2]. But consuming more fishes may be a great concern of food security as it has been known from the data of CDC, USA during the period between 1998 and 2015 that 3% people of United States acquired foodborne illnesses from contaminated fish [3]. FAO reported each year in Bangladesh nearly about 30 million people are affected with food-borne diseases [4]. Though the involvement of fish causing food-borne illness is not clear in this tropic, several microorganisms that are normal inhabitant in aquatic environment may cause contamination [5].

Due to the relatively dense population and poor sanitation facilities, the people of Bangladesh are more susceptible to microbial attack and facing the challenges of faecal contamination [6]. A large group of population who are involved in unhygienic sanitary practice mostly live beside the haor, baor, beel, pond and river. The disposal of inadequate treated or untreated domestic sewage into these natural water bodies, the place of fish harvest, may contain several human pathogenic microbes [7]. In addition, the surroundings of domestic local markets which remain most of the cases soggy, filthy and unhealthy may exacerbate the situation [8]. Unhygienic

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landing sites, poor handling, transport, storage, display and packaging process may increase the risk of microbial contamination of fish from different sources [9]. As a consequence, consumers may develop infection or intoxication [10]. Moreover, ingestion of contaminated fish not only involved food safety issues but also increasing of antimicrobial resistance is remarkable public health crisis [11]. 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, which is a matter of great concern [12].

A member of coliform group, *Escherichia coli* is not only found utterly in the intestinal tract of the animals and humans but also a natural dweller of the fish microbiota [13]. It is widely accepted faecal contamination indicator organism of fish and water [14]. It can be easily propagated in various living ecosystems, interchange genetic material to other bacterial communities that may lead to the development of resistant bacteria, ultimately causing the disease of humans [15]. A higher occurrence of resistant *E. coli* strains has been noticed in fish and seafood in India [16] and Korea [15] indicates the urgency of the monitoring the presence of the bacteria [17]. A limited study was 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 are following seven different categories: Koi (*Anabas testudineus*), Poa (*Otolithoidespama*), Loitta (*Harpadonnehereus*), Sorputi (*Puntiussarana*), Rupchanda (*Pampuschinensis*), Bhata (*Labeobata*) and Taki (*Channa punctate*). Each type of fish samples was aseptically collected after one week interval into sterile plastic boxes, afterwards placed them in 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 faecal coliform count (TFCC).

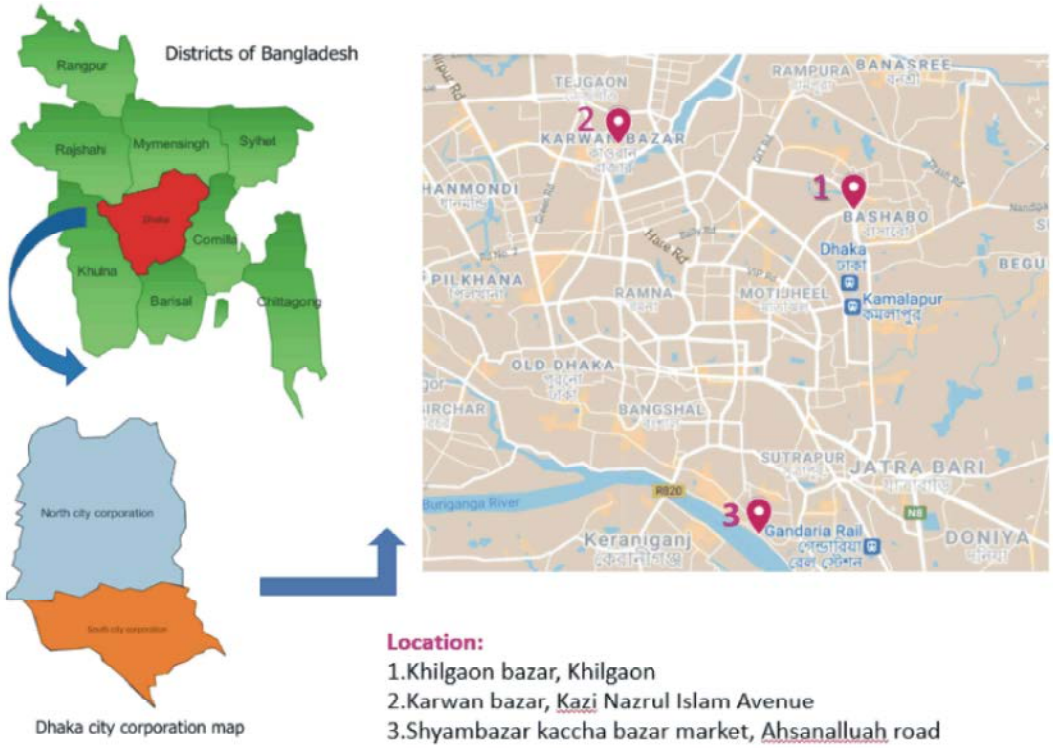

Fig. 1: The map of Dhaka city indicates the area of sampling sites

Sample Processing and Media Preparation: About 20 g muscle of individual fish samples were weighed aseptically in an analytical balance (Shimadju, Japan) in triplicate fashion. Thereafter 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 medium used in this study including nutrient agar, lauryl tryptose broth (LTB), 2% brilliant green bile broth (BGBB) and MacConkey broth were prepared according to Cheesbrough [18]. All the media were autoclaved at 121°C for 15 min prior to use.

Quantitative Analysis of Total Viable Bacteria: Aerobic plate count (APC) method was applied for the estimation of the number of total microbial load [19]. 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 samples 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.

Estimation of Total Coliforms and Faecal Coliforms: Lauryl tryptose broth (LTB), 2% brilliant green bile broth (BGBB) and MacConkey broth were used to estimate the number of total coliforms and faecal coliforms. Most Probable Number (MPN), the most conventional 3-tube method was employed here according to standard protocol [19]. A similar decimal serial dilution series for each sample was performed here (i.e., 9 ml LTB plus one 1 mL crushed fish samples) as earlier. 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 set respectively and incubated at 37°C for 24-48 hours. The indicator, gas and acid produced from lactose fermentation point out presumptive heterofermentive metabolism of coliforms [19]. To confirm the presence of total coliforms and faecal 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°C for 48 hours and MacConkey broth (Oxoid, Uk) at 44.5±0.5°C for 48 hours respectively. In both cases, the gas produced in the tubes after incubation were recorded and compared with the MPN chart to count the total coliforms and total faecal coliforms number.

Isolation and Identification of *E. coli*: From each gas positive MacConkey broth, one loopful faecal coliforms sample was streaked on eosin methylene blue (EMB) agar (Scharlau, Spain) plate. The plates were incubated at 37°C and examined after 24-48 hours for bacterial growth and any change of the colour in the medium. Black colonies with a metallic green sheen were observed on 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* [19].

Antimicrobial Susceptibility Testing: The bacterial isolates were subjected to culture sensitivity test on Muller-Hinton agar (MHA) (Hi-Media, India) plate according to Kirby-Bauer disc diffusion method [20]. A total of twelve panels of commercial antibiotic disks (Oxoid, UK) were used at different concentrations. The disc strength were: Cefixime, Rifampicin and Ciprofloxacin at 5 µg/ml; Gentamicin, Ampicillin, Penicillin, Streptomycin and Norfloxacin at 10 µg/ml; Erythromycin at15 µg/ml and Tetracycline, Chloramphenicol and Neomycin at 30 µg/ml. Isolates were recorded as sensitive, intermediate or resistant on the basis of zone of inhibition in compliance with the criteria of Clinical Laboratory Standards Institute [21]. Further, multiple antibiotic resistance (MAR) index of only multiple drug resistant (MDR) *E. coli* isolates was calculated. Krumperman [22] represent 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 outcomes and p-value $\leq$ 0.05 was considered as significant for mean values.

RESULTS

The average total viable bacterial count (TVBC) of all samples was 6.30 $\log_{10}$CFU/g, ranging from 4.08-6.47 $\log_{10}$ CFU/g. The mean value of total viable bacterial count belonged to seven different fish species were 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.

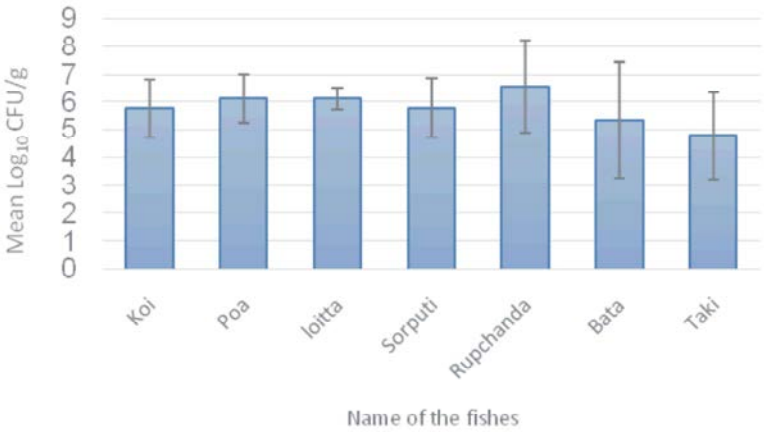

Fig. 2: Total viable bacterial count (log_{10} CFU/g) of the raw fish samples collected from local markets. The values were represented with mean ± SD

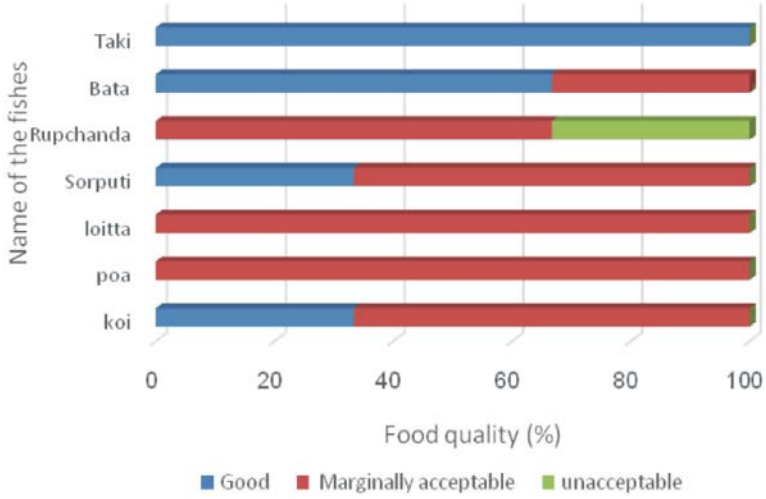

Fig. 3: Microbiological quality of the fishes according to International Commission on Microbiological Specifications for Food (ICMSF)

Table 1: The Total Coliforms (TC) and Total Faecal Coliforms (TFC) count assessment of raw fish samples

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

Based on ICMSF, 1986 criteria, 100% samples of Taki were good microbiological limit. In contrast, cent percent Loitta and Poa fishes and 66.67% Rupchanda fishes were marginally acceptable quality (Fig. 3). Notably, 33.33% Rupchanda fishes with unacceptable limit were noticed.

Nearly all the representative fish samples were contaminated with coliforms and faecal coliforms bacteria. The bacteriological quality of the fish samples brought from the local markets was summarized in the Table 1. In the present research work, the total coliforms count

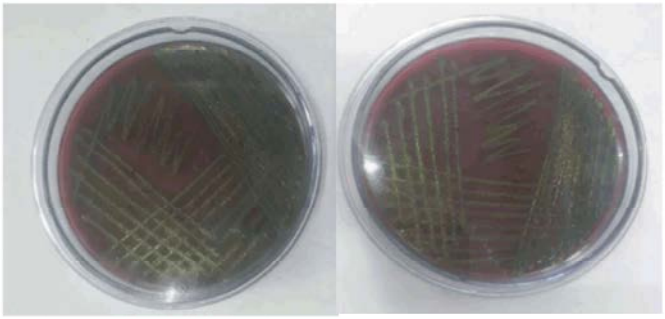

Fig. 4: Growth of *E. coli* colonies on EMB agar plate

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

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

where + = positive and - = negative

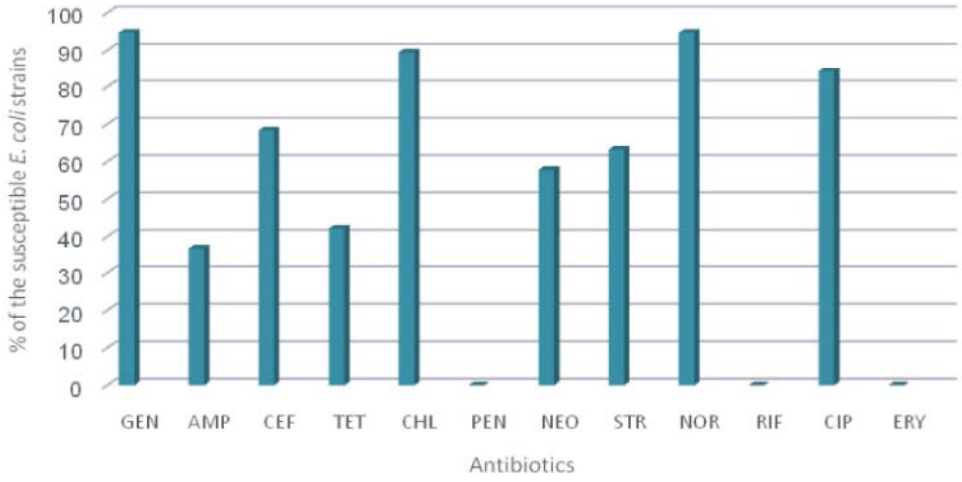

Fig. 5: The percentage of susceptible *E. coli* isolates to multiple antibiotics. The isolated strains were highly susceptible to GEN and NOR while resistant to PEN, RIF and ERY. Here, GEN=Gentamicin, AMP=Ampicillin, CEF=Cefixime, TET=Tetracycline, CHL=Chloramphenicol, PEN=Penicillin, NEO=Neomycin, STR=Streptomycin,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

(TCC) was ranged between 2.1 and >1100 MPN/g and total fecal coliforms count (TFCC) were 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.

Following the conventional culture technique, 19 out of 21 samples displayed black colonies with a metallic green sheen on 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). The overall prevalence of *E. coli* in the present research finding was more than 90%. The presence of the organism in the fish was 100% (21 of 21) in the two sampling sites Shyambazar and Karwan bazar. Moreover, lower contamination rate was reported in the Khilgaon bazar that was 71.4% (19 out of 21).

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

Table 3: 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

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

No. of isolates	% of isolates	No of antibiotics to which the isolates were resistant (a)	MAR Index
4	21.05	3	0.25
2	10.53	4	0.33
4	21.05	5	0.42
6	31.58	6	0.5
1	5.26	7	0.58
1	5.26	8	0.67
1	5.26	9	0.75

Pattern of Antibiotic Resistant Isolates: The antibiotic susceptibility tests revealed that all of the *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* were displayed in Table 3.

Table 4 depicts the summary of the MAR index value which varied within the range of 0.25 to 0.75. Maximum 31.58% 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 MAR index above 0.2.

DISCUSSION

Fishes are becoming popular for their exceptional health benefits. But contamination of fish with bacterial pathogens could create severe foodborne infections and offset the health facilities. In our country, contamination of fresh fish is expected to occur due to densely living population around the river bank, untreated sewage and improper sanitation facilities.

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 their criteria, the TVB count of the 33.33% (7/21) samples were within the range of good quality whereas 61.91% (13/21) and 4.76% (1/21) samples fell into marginally acceptable ($P<0.05$) and unacceptable limit respectively. Hence, statistically significant percentages of the samples, 66.67% (14 out of 21) were beyond the good quality limit ($p<0.05$). Several previous investigations performed on different variety of fishes from retail shops also reflect the same scenario. The TVBC ranging from 6×10^4 to 1.6×10^6 CFU/g [23], 2.89×10^5 to 2.98×10^7 CFU/g [8] and 3.3×10^5 CFU/g to 1.9×10^8 CFU/g [24] comply with the outcomes of the present study. So, the samples of domestic markets comprised of high bacterial load may be transmitted to the consumers and the people associated with the transportation, handling and processing through contact [25]. 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.

The faecal contamination of fishes and natural water bodies is a common trouble in developing countries. ICMSF establish the standard acceptance limits of total coliforms (TCC) and fecal coliforms (TFCC) for fresh fish, that are <100MPN/g and <10MPN/g respectively.

The recent study found that almost 66.67% (14 of 21) and 95.25% (20/21) examined species exceeded the limit of acceptance in a significant way ($P<0.05$) for coliforms and fecal coliforms respectively. Moreover, the foremost faecal coliforms indicator *E. coli* was observed around 90% samples may indicate the probability of the presence of these bacteria in higher density in fresh water. High *E. coli* load like 80.70%, 46.6% and 80% was also found in India [7] Kenya [26] and Switzerland [17] respectively. The prevalence of coliforms and faecal coliforms at higher ranges may point out the 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 [27].

From the report of the antibiotic resistance study performed on all of the *E. coli* isolates; it was known that they were resistant to at least three antibiotics. Cent percent strains harbored resistance to rifampicin, penicillin and erythromycin. This observation was nearly concordant to the investigations of Pinu *et al.* [28] and Ahmed *et al.* [29] where strains of *E. coli* harvested from different fish species exhibited resistance to penicillin G and erythromycin respectively. The origins of these drug resistant *E. coli* isolates might come from hospital or veterinary source though the data concerning the prevalence of antibiotic resistant *E. coli* strains either in clinical or environmental sources are not available in Bangladesh.

Moreover, in our country, antibiotics are indiscriminately used in agriculture and animal industry. Adeleke & Omafuvbe [30] calculated a MAR index value that clarifies the potential health risk among the population whether the distribution of resistant bacteria in a particular population is high or low. The MAR value greater than 0.2 indicates, the strains of particular bacteria evolved from an environment in which multiple antibiotics are frequently used. Nearly all the samples (100%) of the recent study exceeded the threshold MAR index value that was in the range of 0.25 to 0.75; reflect a scenario that somehow there is a misuse or overuse of antibiotics in fish farms and related water bodies. In fact, it is really hard to predict the source and routes of contamination until an 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 the transmission of food borne disease has already been reported [31]. In our region, food-borne illness related to fresh fish consumption has not yet been traced out or data on this issue is still lacking. In this respect, bacteriological assessment of local fishes appears to be a foremost issue. This kind of study generates scientific facts which would help the authority to take proper steps to maintain the microbial quality of the fishes as well as hygiene of fish markets and aquatic environments.

In conclusion, this comprehensive study reported the poor hygiene status of our domestic markets and pitiable quality of fishes 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. In addition, to find out the source of contamination and routine examination of other foodborne pathogens related with fish shall be the focus of the future research.

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