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SURVEILLANCE OF *ESCHERICHIA COLI* IN A FISH FARM OF SYLHET, BANGLADESH

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Abstract: The study was accomplished to investigate *Escherichia coli* from two freshwater fish, Tilapia (*Oreochromis niloticus*) and Mrigal (*Cirrhinus mrigala*), collected from a fish farm in Sylhet, Bangladesh. Six of each fish were analyzed to isolate and detect *Escherichia coli*, and *E. coli* was identified based on morphological and biochemical characteristics. The antibiogram of *E. coli* was investigated in different generations using eight antibiotic discs such as Chloramphenicol (CH), Streptomycin (S), Gentamycin (G), Ciprofloxacin (CI), Co-trimethoxazole (CO), Azithromycin (AZI), Erythromycin (E) and Novobiocin (NV), and the sensitivity of *E. coli* was found as 100%, 25%, 100%, 75%, 87.5%, 81.25%, 0%, 0% respectively. Among the 8 antibiotics, for Erythromycin (E) and Novobiocin (NV), the observed resistance pattern of *E. coli* was 81.25% and 87.5% respectively, whereas, for the rest of the antibiotics, it was 0%.

Key words: freshwater, aquaculture, *Escherichia coli*, antibiotic resistance

INTRODUCTION

Bangladesh is rich in fisheries resources and recognized as the third aquaculture country in the world (FAO 2018), including vast culture potential where the inland aquaculture sector is contributing more than 55% of the total production of Bangladesh (DoF 2016). Among 266 freshwater fish including16 exotic species, around 25 species are being cultured (IUCN 2014, FRSS 2016). Freshwater aquaculture is mainly comprised of pond farming in Bangladesh where Tilapia (*Oreochromis niloticus*) and Mrigal (*Cirrhinus mrigala*) are commonly cultured species. However, among the other causes, diseases are responsible for the huge loss of production in aquaculture, especially in developing countries as they contain 90% of the aquaculture farm. For example, in Chile, 2 billion dollars lost caused by infectious salmon anemia alone and

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15% loss of total fish production to diseases in China (Leung *et al.* 2013 and Assefa *et al.* 2018).

Different pathogenic bacteria are involved to impact the fish production in Bangladesh whereas few fecal coliforms were observed (Mandal *et al.* 2009) as a fish pathogen that often contaminants of food and water through *E. coli* is mostly non-pathogenic (Dutta *et al.* 2010, Soliman *et. al.* 2010, Eze *et al.* 2011). Generally, for the potential sewage pollution, based on strains, *E. coli* present in fish are considered as an indicator (Hanson *et al.* 2008). Inside the intestine of fish, *E. coli* commonly resident as non-pathogenic but when expanding outside the intestine, it can be responsible for causing disease, resulting in entero-toxigenic (Lee and Marks 2009) whereas 18 toxigenic *E. coli* were isolated (Vieira *et al.* 2001). The sensitivity of different antibiotics was examined and found high effect against *E. coli* isolates from fish samples (Sukumaran *et al.* 2012, Soliman *et al.* 2010). The reason for the versatility of *E. coli* strains of involving different strains with different diseases is because they obtaining virulence genes of different sets. *E. coli* strains are related with different disease-causing fish virulence genes as well (Teophilo *et al.* 2002, Gupta *et al.* 2013).

As a remarkable number of fish species are available in the freshwater of Bangladesh, tilapia (*O. niloticus*) and mrigal (*C. mrigala*) contribute an important role in nutrition as well as the national economy. Though few researches observed the occurrence of fecal coliform mostly in *E. coli* of Nile tilapia (Thampuran *et al.* 2005, Mandal *et al.* 2009). However, information is not available for mrigal (*C. mrigala*) in Bangladesh. Due to the high economic value of these two fishes in Bangladesh, this research is aimed to observe the status of fecal coliform in a farm condition of Sylhet and to know the surveillance of *E. coli* in terms of occurrence, detection, and antibiogram assay.

MATERIAL AND METHODS

Sample collection: The study was conducted in the Department of Genetic Engineering and Biotechnology (GEB) at Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh. Total 12 fish of Tilapia (*Oreochromis niloticus*) and Mrigal (*Cirrhinus mrigala*), 6 for each were collected from a farming pond of Sylhet, Bangladesh, which then immediately transported to the Zoology, Fisheries and Marine Biotechnology Research Unit in the Department of GEB, SUST for further studies. Fish samples were identified according to the morphometric characteristics (Shafi and Quddus 1982, Rahman 1989) and *E. coli* was isolated from the liver, heart, gills, scales surface, and fin of the fish samples.

Preparation of experimental materials: The isolation of the bacteria from experimental fish and identification of *E. coli* isolates was performed using morphological and biochemical characteristics. All materials like Petri dishes, test tubes, stock bottles, etc. were washed with detergent and dried at 70^0C in an oven drier and sterilized at 170^0C for 1.5 hours by a hot air sterilizer. The tips were washed and autoclaved at 121^0C for 15 minutes. Then the glassware was again put into a drier at 70^0C before use. Most of the sterilization processes were followed according to the instructions described by Barrow and Feltham (1993).

Isolation of E. coli by selective culture media: First of all, 51.53 grams of MacConkey agar was added to a flask containing 1000 ml of distilled water, and to dissolve the medium completely, the heat was applied until the boiling. Through the autoclave, the medium was subjected to sterilization. Furthermore, 38 grams of EMB agar base (Himedia, India) also added to a flask containing 1000 ml of distilled water and for a fine mixture, the heat was applied. Again, for sterilizing the medium, autoclave was done at 121^0C (15 psi) for 15 minutes and then, to lowering the temperature of the medium, it was placed into a water bath of 45^0C. When the medium was solidified, to confirm the sterility, the Petri dishes were kept incubated at 37^0C overnight and then stored at 4^0C in the refrigerator until used. The samples collected were placed on a MacConkey agar plate by using a sterile loop and cultured on the medium by the streaked plate technique, which were then incubated at 37^0C for 24 hours. The appearance of the colonies a characteristic green metallic sheen with a dark center was considered positive for *E. coli.*

Performance of pure culture of E. coli: About 500 ml of Nutrient Agar (NA) medium was prepared followed by Cappuccino and Sherman (2007) and stirred by a magnetic stirrer. Following the previous autoclave procedure, the medium was sterilized and after reducing the temperature of the medium up to 60˚C, it was poured into sterile Petri dishes with the amount of 25 ml each, which are then kept for future use after the solidifying of the plates. A single colony was taken from EMB agar plate by using sterile loop and inoculated on the NA medium. The plates were then incubated at 37^0C for 24 hours.

Morphological Identification of E. coli strains: The bacterial colonies grown on agar medium were recorded for their colony characteristics such as size, shape, the color of the colony. Morphological characters of the isolates such as shape and size were recorded during Gram's staining procedure with the fresh subculture of 24 hours and recorded accordingly. To differentiate the motile bacteria from the non-motile ones, the motility test was done followed by the

method mentioned by Cowan (1985) where observing the movement of bacteria, motility were identified.

Gram staining was conducted followed by the method described by Petersen *et al.* (2016) using a compound microscope.

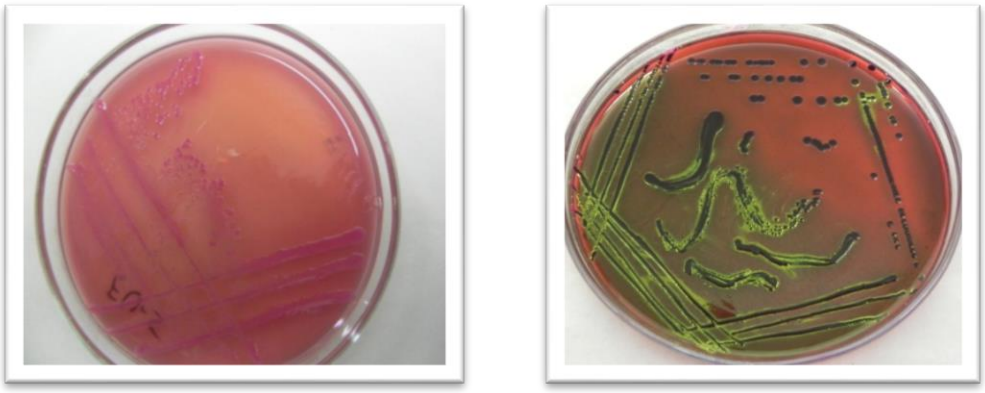
Biochemical identification of E. coli isolates: For the verification of *E. coli*, the biochemical tests performed were Voge's Proskauer (VP) test, methyl red (MR) test, Catalase test, TSI test, Oxidase test, urease activity test, lactose fermentation tests, indole test, and Citrate utilization test. *E. coli* were distinguished by their ability to ferment lactose, positive for indole test and MR test and negative for VP, urease activity, Oxidase test, and Citrate utilization test.

Preservation of bacterial isolates in pure culture form: To use the prepared pure culture as a stock culture, it was stored at -20°C after adding equal volume of 80% glycerin.

Detection of Antimicrobial Sensitivity Patterns: Kirby-Bauer method of antibiotic susceptibility testing (AST) was used to study *E. coli* which is known as the disk diffusion method as well. In this experiment, we used the discs produced by Himedia Laboratories Pvt. Limited, Mumbai. The procedure of the antimicrobial sensitivity was used for 15 isolated *E. coli*.

Susceptibility test of identified *E. coli* and ATCC was done on Mueller Hinton Agar and 8 different antibiotic discs such as Streptomycin, Erythromycin, Gentamycin, Chloramphenicol, Ciprofloxacin, Co-trimethxazole, Azithromycin, and Novobiocin were used. After preparing the media with proper manufacture's instruction, it was sterilized by autoclaving. Sterilized Petri dishes containing media were allowed to solidify. The fresh nutrient broth was prepared and a volume of 6-8ml was dispensed in each test tube which then sterilized by autoclaving. Identified *E. coli* and ATCC strains were then inoculated with a sterilized loop from pure culture and were incubated for 24 hours at 37°C.OD_{600} was measured by using a spectrophotometer for keeping the same bacterial density and the OD_{600} of broth cultures were adjusted at 0.24 by serial dilution method. By using L shaped glass rod, 100 microliters of microbial inoculums from the diluted nutrient broth was poured and spread throughout the surface of Mueller Hinton Agar. The antibiotic disc was set on the surface of the ager, with the help of a sterilized needle at a maximum distance for getting distinct zones. The Petri dishes were then subjected for incubation at 37 °C for 12-18 hours. In this method ATCC strain (25922) was used as a reference. By analysis with the standard table for the zone of inhibition, all the results were detected. The Petri dishes were examined after 12-18 hours and then calculated the zone

of complete inhibition (mm in diameter). From the analyzed data, we can get the % of resistance, intermediate, and sensitivity pattern of the identified *E. coli*.



(a) (b)

Fig.1: Identification of *E. coli:* (a) *E. coli* culture on Mac Conkey's agar plate; b) *E. coli* culture on EMB agar plate.

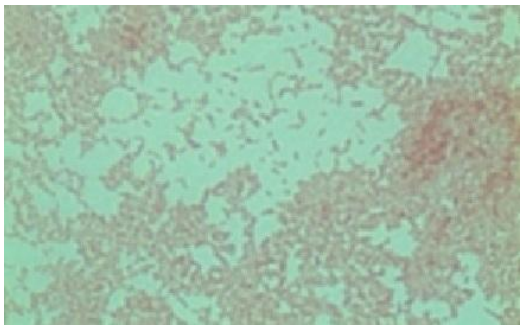


Fig. 2: Gram's staining of bacterial isolates showing Gram-negative , small rod-shaped, pink coloredand single or paired organisms

RESULTS AND DISCUSSION

Isolation and identification of E. coli: A total number of 12 fish samples were collected from the different markets of Sylhet city and then screened for the confirmation of *E. coli* (Fig. 1). All the examined Tilapia and Mrigal samples were found positive with *E. coli*. A total 21 of *E. coil* isolates were isolated from 12 experimental fish samples. On the basis of samples examined, the incidence of *E. coli* varies.

Motility test: Twenty-one isolates were found motile in the motility test.

Gram's stain: In Gram's staining, the organism showed gram-negative, small rod-shaped, pink color, and single or paired arrangement characteristics (Fig. 2) under the microscope.

Biochemical tests: For the suspected bacteria, several biochemical tests were conducted for characterization. All biochemical test results are given in Table 1.

Table 1. Biochemical test for *E. coli* identification

Sample code	M	G	MR	VP	C	TSI	O	U	LF	IT	CU
E-1	+	-	+	-	+	+	-	-	+	+	-
E-2	+	-	+	-	+	+	-	-	+	+	-
E-3	+	-	+	-	+	+	-	-	+	+	-
E-4	+	-	+	-	+	+	-	-	+	+	-
E-5	+	-	+	-	+	+	-	-	+	+	-
E-6	+	-	+	-	+	+	-	-	+	+	-
E-7	+	-	+	-	+	+	-	-	+	+	-
E-8	+	-	+	-	+	+	-	-	+	+	-
E-9	-	+	-	-	+	-	+	-	+	-	-
E-10	+	-	+	-	+	+	-	-	+	+	-
E-11	+	-	+	-	+	+	-	-	+	+	-
E-12	+	-	+	-	+	+	-	-	+	+	-
E-13	+	-	+	-	+	+	-	-	+	+	-
E-14	+	-	+	-	+	+	-	-	+	+	-
E-15	+	-	+	-	+	+	-	-	+	+	-
E-16	+	-	+	-	+	+	-	-	+	+	-
E-17	-	-	+	+	-	+	-	+	-	+	+
E-18	+	-	+	-	+	+	-	-	+	+	-
E-19	+	+	-	+	-	+	+	-	-	-	+
E-20	-	-	+	+	-	-	+	+	-	-	+
E-21	-	+	-	-	+	-	-	+	-	+	+

M= Motility, G= Gram test, MR= Methyl Red test, VP= Voge's-Proskauer, C= Catalase test, TSI= Triple Sugar Iron, O= Oxidase test, U= Urease Activity, LF= Lactose Fermentation Test, IT= Indole Test, CU= citrate agar test.

Presumptive result: To analysis, all *E. coli* isolates, it was confirmed that E1, E2, E3, E4, E5, E6, E7, E8, E10, E11, E12, E13, E14, E15, E16, and E18 isolates were *E. coli*. *E. coli* was observed as Oxidase negative, Catalase positive, Gram-negative, Indole test positive, MR positive, VP negative, and TSI (Triple Sugar Iodine Agar) test positive. List of presumptive *E. coli* isolates was identified according to Bergey's (1994) and Cowan & Steel (1974).

Determination of antibiotic sensitivity pattern: 16 isolates were screened for drug resistance profile. All the isolates showed the highest degree of sensitivity against the commonly used antibiotics (Table 2). *E. coli* ATCC strain was found

to be highly sensitive against the used antibiotics. Table 3 showed the Standard clear zone diameters for *Enterobacteriaceae* and table 4 showed the result of this research. The result investigate that sensitivity pattern of *E. coli* for 8 antibiotics {Chloramphenicol (CH); Streptomycin (S); Gentamycin (G); Ciprofloxacin (CI); Co-trimethoxazole (CO); Azithromycin (AZI); Erythromycin (E); Novobiocin (NV)} were respectively 100%, 25%, 100%, 75%, 87.5%, 81.25%, 0%,0%. Whereas resistance patterns of *E. coli* for 8 antibiotics were 81.25% and 87.5% for Erythromycin (E) and Novobiocin (NV) respectably. The resistance pattern of *E. coli* for the rest of the 6 antibiotics was 0% to all. On the other hand, intermediate pattern of *E. coli* for 8 {Chloramphenicol (CH); Streptomycin (S); Gentamycin (G); Ciprofloxacin (CI); Co-trimethoxazole (CO); Azithromycin (AZI); Erythromycin (E); Novobiocin (NV) } antibiotics were respectively 0%, 75%, 0%, 25%, 12.5%, 18.75%, 18.75%, 12.5%.

Table 2. Antibiotic sensitivity and resistance pattern of isolated *E. coli* by measuring zone diameter of inhibition

Sample code	S	E	G	CH	CI	CO	AZI	NV
E-1	15mm	15	23mm	25mm	21mm	26mm	18mm	NZ
E-2	20mm	6mm	20mm	22mm	24mm	20mm	20mm	6mm
E-3	18mm	NZ	22mm	25mm	20mm	22mm	19mm	12mm
E-4	20mm	14mm	24mm	20mm	28mm	18mm	22mm	NZ
E-5	19mm	10mm	21mm	27mm	22mm	14mm	16mm	NZ
E-6	22mm	NZ	20mm	22mm	25mm	24mm	18mm	8mm
E-7	20mm	16mm	21mm	28mm	28mm	18mm	20mm	NZ
E-8	20mm	NZ	22mm	22mm	24mm	28mm	18mm	10mm
E-10	22mm	NZ	24mm	23mm	20mm	20mm	20mm	NZ
E-11	18mm	6mm	20mm	26mm	23mm	16mm	18mm	13mm
E-12	17mm	NZ	23mm	26mm	20mm	20mm	21mm	NZ
E-13	18mm		20mm	24mm	26mm	15mm	17mm	NZ
E-14	22mm	NZ	21mm	20mm	22mm	20mm	21mm	7mm
E-15	14mm	8mm	20mm	23mm	20mm	16mm	16mm	7mm
E-16	20mm	9mm	22mm	20mm	23mm	22mm	20mm	NZ
E-18	21mm	NZ	24mm	25mm	21mm	18mm	18mm	9mm

Note: S = Streptomycin; E = Erythromycin; G = Gentamycin; CH = Chloramphenicol; CI = Ciprofloxacin; CO = Co-trimethoxazole; AZI = Azithromycin; NV = Novobiocin

The study was conducted to investigate the prevalence of *Escherichia coli* in freshwater fish particularly in Tilapia (*Oreochromis niloticus*) and Mrigal (*Cirrhinus mrigala*) in Sylhet, Bangladesh.

In this study, with the nutrient broth turbid, black centered colony with metallic sheen was observed in EMB agar. The present results similar to the findings of the Zinnah *et al.* (2007) and Sarba *et al.* (2019) presumptively recognized *E. coli* where they found in an EMB agar a greenish-black colony along with a metallic sheen. Same results were also found by some other

authors (Buxton and Fraser, 1977; Freeman, 1985; Jones, 1987) whereas characteristic features of *E. coli* such as gram-negative, pink color, small rod-shaped were noticed in gram's staining as well as hanging drop technique (Cowan, 1985) in which all the isolates showed motility. At the same time, the study possess the similar findings of Zinnah *et al.* (2007), Sarba *et al.* (2019), and Buxton and Fraser (1977) where the yellow slant revealed from the TSI agar slant with no hydrogen sulphide production. *E. coli* was confirmed by several biochemical tests were according to the Ali *et al.*, (1998).

Table 3. Standard clear zone diameters for *Enterobacteriaceae*

Name of the antibiotics	Zone diameter (mm)		
	Resistant	Intermediate	Sensitive
Azithtromycin (AZI)	≤13	14-17	≥18 -
Ciprofloxacin (CI)	≤15	16-20	≥21
Gentamycin (G)	≤12	13-14	≥15
Erythromycin (E)	≤13	14-22	≥23
Co-Trimoxazale (Sulpha/ Trimethoprim) (CO)	≤10	11-15	≥16
Streptomycin (S)	≤13	14-20	≥21
Chloramphenicol (CH)	≤12	13-17	≥18
Novobiocin (NV)	≤11	12-15	≥16

Table 4 Antimicrobial susceptibility pattern of *E. coli* isolated

Name of Antibiotics	Antibiotic conc. (µg/disc)	Sensitivity pattern of isolated *E. coli* strains			Sensitivity pattern of *E. coli* ATCC strain		
		%R	%I	%S	%R	%I	%S
Streptomycin	10	-	75	25	-	-	100
Erythromycin	15	81.25	18.75	-	100	-	-
Gentamycin	10	-	-	100	-	-	100
Chloramphenicol	30	-	-	100	-	-	100
Ciprofloxacin	5	-	25	75	-	-	100
Co-trimethxazole	25	-	12.5	87.5	-	-	100
Azithromycin	30	-	18.75	81.25	-	-	100
Novobiocin	30	87.5	12.5	-	100	-	-

These days, antimicrobial resistance became a worldwide matter of concern (Islam *et al.* 2016 and Miles *et al.* 2006). In country like Bangladesh, the issue of multi-drug resistant strain of *E. coli* is constantly raised due to the abuse of antibiotics (Islam *et al.*, 2016). Although this finding was found to be similar with the many past studies, the incident was increased in food as well as human beings by several factors such as use of non-selective antibiotics with insufficient knowledge and at the same time carelessness towards the disease. In the present study, the prevalence, pattern of antibiotic sensitivity of *E. coli* in fish were examined.

Spreading of the drug-resistant bacteria in the sewage and surface water occurred due to the release of human, animal, and bird fecal. Under some defined physicochemical and biological conditions, the resistant *E. coli* strains interchange R-plasmids to susceptible strains of *E. coli* (Rahman, 2008). When the environment get contaminated with the drug-resistant *E. coli*, human as well as animals face difficulties in the treatment after getting the infection (Joseph *et al.*, 1979).

Multiple-drug (Erythromycin and Novobiocin) resistance was seen in all the strains and there is a common resistance pattern for antibiotics used among the strains. The mechanism for the spread of antibiotic resistance must be considered seriously like this present study. It is an alarming sign that the percentages of sensitivity towards all the randomly selected six antibiotics werenot satisfactory. From all of these antibiotics, Erythromycin (E) and Novobiocin (NV) were found unable to inhibit bacterial growth so that we cannot use them for the treatment of various diseases caused by *E. coli* from freshwater fish. At present, the possible reason of this high incidence of multidrug resistance is the non-selective use of antibiotics. With time, this might be taking the place of drug-sensitive organisms from the antibiotic saturated environment(Jawetz *et al.,* 1984).

Resistance to Erythromycin is becoming a serious clinical problem (Mycek *et al.*, 2000). In this study, *E .coli* was found the height resistance to the Erythromycin (81.25%) while Al-Ghamdi *et al.*, (2001) found *E. coli* isolates from layer and broiler source resistance to Erythromycin (87.4%).

E. coli is a common pathogen isolated from fish meal and fish farming water (Oliveira *et al.* 2017 and Ristori*et al.*2007), and play an important role in economic losses among the fish industry. As *E. coli* is highly pathogenic and cause diarrhoea (Sousa, 2006), so special care and further investigation are required to tackle this situation. Moreover, it is well evidence that *E. coli* causes food poisoning and food spoilage (Ekici *et al.* 2019). Therefore, the present study was performed to isolate and confirmed the presence of *E. coli* in freshwater fish by using various methods. From this study, it appears that *E. coli* collected from freshwater fish samples have some differences in antibiotic sensitivity patterns. Therefore, further study should include a wide variety of molecular tests with a large number of freshwater fish samples to see the difference and variation between them to reach a conclusive finding.

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