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Molecular characterization and antimicrobial resistance among diarrheagenic and non-diarrheagenic *Escherichia coli* from healthy and diseased freshwater farmed fish

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The aim of this study is to understand molecular characterization and antimicrobial resistance of diarrheagenic and non-diarrheagenic *E. coli* from healthy and diseased farm fish from *Cypriniformes* order. A total of 516 healthy and diseased farmed fish samples belong to *Cypriniformes* order were collected from various regions of Punjab and screened for *E. coli*. Biochemical tests and PCR confirmation were conducted using the *16S rRNA* and *Usp*A genes. DEC was examined through PCR using different virulence genes. All isolates were tested for various antibiotics using the disk diffusion method. The phenotypic and genotypic resistances of Extended-spectrum β-lactamases (ESBLs) were investigated. A Chi-square test was performed to compare the prevalence of *E. coli*, virulence genes, and ESBLs genes with a significance level of $P \leq 0.05$. Out of the total fish samples, 16.66% and 36.57% of *E. coli* strains were confirmed through biochemical tests and PCR, respectively. The DEC pathotypes were recorded in 30% and 62.02% of isolates from healthy and diseased fish, respectively. A higher rate of 19.37% of EAEC pathogroup was observed in both healthy and diseased fish, while the EIEC pathogroup was only observed in diseased fish at a rate of 10.07%. The significance ($P \leq 0.05$) was observed among the virulence genes. The highest resistance rates among diarrheagenic and non-diarrheagenic strains were observed with tetracycline, amoxicillin, and ceftazidime. A higher prevalence of *CTX-M* (56.81%) and *TEM* (43.18%) was recorded in DEC pathotypes, while ^{bla}*SHV* was not detected. The high prevalence of DEC *E. coli*, and the production of ESBLs, indicate unhygienic practices and the misuse of antibiotics in fish farms.

Keywords: Carps, *E. coli*, Virulence genes, Extended-spectrum Beta-lactamases

INTRODUCTION

The increasing demand for aquaculture products has driven a shift from extensive systems to more intensive and semi-intensive systems in order to achieve higher yields (Dawood and Koshio, 2016). However, this intensification often leads to deteriorating water quality, which can result in pathogenic outbreaks and, consequently, have detrimental effects on production and compromised product quality (Chen *et al*., 2014). Fish-associated bacteria can be classified as nonindigenous and indigenous. Nonindigenous bacteria include *E. coli*, *Salmonella*, *C. botulinum*, *S. dysenteriae*, *S. aureus*, and *L. monocytogenes*. These nonindigenous bacterial pathogens commonly contaminate fish and their habitats through various means (Marijani, 2022).

Escherichia coli is a facultative anaerobic bacterium commonly found in the gastrointestinal tract (GIT) of monogastric and ruminant animals. While it is typically harmless, *E. coli* can also be a medically significant bacterium, causing numerous illnesses in both humans and animals. This bacterium is frequently associated with fecal contamination in aquatic environments and fish (Costa, 2013). It is classified into various pathotypes based on their potential virulence genes.

Six different pathotypes of *E. coli* have been well described, each capable of causing intestinal diseases: diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) (Kaper, 2005). These pathotypes have been documented in various aquatic sources

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such as fish, shrimps, and fish meal (Ristori *et al*., 2007; Surendraraj *et al*., 2010). Studies have investigated the prevalence of *E. coli* strains in different fish species, including farmed *Oreochromis niloticus* and *Cirrhinus mrigala*, as well as wild fish (Hanson *et al*., 2008; Reza *et al*., 2020). Additionally, *E. coli* has been detected in sediment, water, soil, sand, fish meal, fish farms, lakes, and freshwater shellfish found in markets and farms (Thampuran *et al*., 2005; Ishii and Sadowsky, 2008, Manna *et al*., 2008; Assefa *et al*., 2019; Cheng *et al*., 2019). To mitigate the occurrence of such catastrophes, antibiotics are often used as growth promoters and prophylactic agents in aquaculture. Unfortunately, these practices are particularly prevalent in countries with lax regulations on antibiotic use, as highlighted by (Pruden *et al*., 2013). However, due to the widespread misuse of antibiotics, bacteria have developed increasing levels of resistance, including resistance to multiple drugs, and possess antimicrobial resistance genes (AMR). This alarming situation has made aquaculture a major contributor to antibiotic resistance issues, posing significant negative consequences for public health (Zhang and Ling, 2020). Moreover, AMR genes are considered environmental pollutants and contribute to the expansion of antibiotic resistance in bacteria (Pruden *et al*., 2006). The presence of antibiotic resistance genes in farm-raised fish has been documented by (Deng *et al*., 2014), and such genes have been found in sediment and water samples collected from fish farms (Xiong *et al*., 2015). As the awareness of "One Health" concerns continues to grow, the aquaculture industry is now recognized as a significant global sector in relation to antimicrobial resistance issues (WHO, 2015; Santos and Ramos, 2018; Stentiford *et al*., 2020).

To ensure the protection of public health, it is crucial to promptly assess microorganisms present in drinking water and raw processed components of aquaculture to prevent outbreaks of microbial contamination. A previous study has highlighted the potential transfer of certain microorganisms, particularly from fish to humans (Kaper, 2004). However, the current knowledge regarding the prevalence of *E. coli*, different pathogroups with their respective virulence genes, and Extended-spectrum Beta-lactamases (ESBLs) in farm-raised fish in Punjab, Pakistan, remains limited. Therefore, the main objective of this research study was to molecularly characterize and determine the prevalence of *E. coli*, including both diarrheagenic and non-diarrheagenic strains, while also examining their resistance patterns against various classes of antibiotics.

MATERIALS AND METHODS

Experimental species and sampling area: A total of 300 healthy fish (50 of each fish species) and 216 diseased fish (36 of each fish species), including *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*, *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*, and *Cyprinus carpio*, were collected from various fish farms located in six different districts of Punjab, namely Muzaffargarh, Gujranwala, Bahawalnagar, Multan, Toba Tek Singh, and Kasur Figure 1 shows sampling sites.

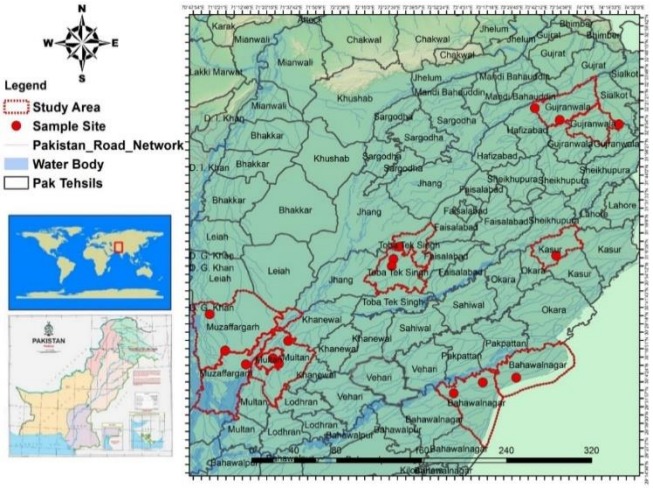

Figure 1. GIS map showing sampling sites

Clinical observation and transportation of fish samples: Clinical observations were documented through consisting of external appearances of fish (Noga, 2010; Eissa, 2016). After documentation of clinical symptoms, fish samples were transported in an icebox (having three layers of ground ice) to the Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore Pakistan, for further examination.

Postmortem examination and conditioning of fish organs: For postmortem examination, the whole fish was sterilized with normal saline except the anus region and kept in sterilized surgical tray. The ventral side of the fish was swabbed with antiseptics and dried with a hygienic gauze pad. Fish body wall was cut with sterilized scissors, the anus and intestine were avoided to cut (Noga, 2010), and the postmortem observations were documented. Further, the fish organs were incubated at 41.5 °C for 6 h to increase the recovery rate of stressed cells (ISO, 2017).

Isolation and biochemical identification of E. coli: The fish organs gut, gills, kidneys, fins, and skin were screened for *E. coli*, followed by homogenization using a homogenizer (SCILOGEX D500, USA). Approximately, 100µl of homogenized tissue samples were cultured on differential media MacConkey agar (Oxoid, UK), as well as on selective Eosin Methylene Blue (EMB) (Oxoid, UK), and incubated for 24h at 37 °C. Bacterial colonies, color, and shape were observed and further identified through the various biochemical tests (catalase, oxidase, indole, and urease tests) as formerly described by (Fattahi *et al*., 2013; Agoba *et al*., 2017; ISO, 2017). Isolates were inoculated from a pure

Table 1: Primers for molecular detection of *E. coli* , virulence and ESBLs genes

Genes	Sequence (5´-3´)	bp	PCR Amplification conditions					Cycle	References
			ID	FD	A	IE	FE		
*Usp*A	F: CCGATACGCTGCCAATCAGT	884	95ºC,	94ºC,	58ºC,	72ºC,	72ºC,	30	Osek (2001)
	R: ACGCAGACCGTAGGCCAGAT		2min	30sec	1min	1min	5min		
16S	F: GGAAGAAGCTTGCTTCTTTGCTG	554	94ºC,	94ºC,	56ºC,	72ºC,	72ºC,	30	Fattahi (2013)
rRNA	R: AGCCCGGGGATTTCACATCTGA		7min	1min	1min	1min	10min		
aggR	aggRKS1: GTATACACAAAAGAAGGAAGC	254	94ºC,	94ºC,	54ºC,	65ºC,	65ºC,	30	Ratchtrachenchai
	aggRKS2: ACAGAATCGTCAGCATCAGC		1min	1min	1min	50sec	50sec		(1997)
IpaH	PS11: TTGACCGCCTTTCCGATACC	647	95ºC,	94ºC,	55ºC,	72ºC,	72ºC,	35	Persson *et al.*
	PS12: ATCCGCATCACCGCTCAGAC		6min	1min	50sec	1min	1min		(2007)
Est	AL65: TTAATAGCACCCGGTACAAGCAGG	147	94ºC,	94ºC,	52ºC,	65ºC,	65ºC,	30	Nayani (2013)
	AL125:CCTGACTCTTCAAAAGAGAAAATTAC		1min	1min	1min	1min	1min		
vtx1	PS3: GTTTGCAGTTGATGTCAGAGGGA	260	94ºC,	94ºC,	54ºC,	72ºC,	72ºC,	35	Persson *et al.*
	PS4: CAACGAATGGCGATTTATCTGC		6min	50sec	40sec	50sec	50sec		(2007)
CTX-M	F: ATGTGCAGYACCAGTAARGTKATGGC	593	95ºC,	94ºC,	52ºC,	72ºC,	72ºC,	35	Monstein *et al.*
	R:TGGGTRAARTARGTSACCAGAAYCAGCGG		15min	30sec	30sec	2min	2min		(2007)
TEM	F: TCGCCGCATACACTATTCTCAGAATGA	445	95ºC,	94ºC,	52ºC,	72ºC,	72ºC,	35	Monstein *et al.*
	R: ACGCTCACCGGCTCCAGATTTAT		15min	30sec	30sec	2min	2min		(2007)
^{bla}*SHV*	F: ATGCGTTATATTCGCCTGTG	747	95ºC,	94ºC,	52ºC,	72ºC,	72ºC,	30	Monstein *et al.*
	R: TGCTTTGTTATTCGGGCCAA		15min	30sec	30sec	2min	2min		(2007)

*bp (Base pair), ID (Initial Denaturation), IF (Final Denaturation), A (Annealing), IE (Initial Extension), FE (Final Extension)

culture in trypticase soy broth and preserved in the glycerol stock at -80°C.

DNA extraction: Genomic DNA of *E. coli* isolates from healthy and diseased fish organs were extracted using DNAzol™ (Catalog number: 10503027, ThermoFisher Scientific, USA) through a prescribed protocol with DNA extraction kit. The DNA quantity and quality were evaluated by spectrophotometer (NanoDrop 1000, ThermoFisher Scientific Inc, USA) (Huang *et al*., 2017) and agarose gel electrophoresis (1% agarose).

Molecular confirmation and detection of virulence genes of E. coli: Species-specific *16S rRNA* and *Usp*A genes (Fattahi *et al*., 2013; Osek, 2001) were used for molecular confirmation of *E. coli* isolates and for molecular confirmation, *E. coli* (ATCC 25922) strain was run as a positive control. All positive *E. coli* isolates were examined for the presence of virulence genes; *vtx1*, *ipaH*, *aggR*, and *est* (Ratchtrachenchai, 1997; Persson *et al*., 2007; Nayani, 2013). PCR amplification was performed in a thermal cycler (Model 1861096, Bio-Rad, USA). The PCR products of 4µl were electrophoresed on 1% agarose (Invitrogen, Thermo Fisher Scientific, USA) stained with 5µl ethidium bromide, and visualized under a Gel Documentation System (Bio-Rad,USA). The primers sequence, PCR amplification conditions and reaction, are listed in Table 1 and 2.

Table 2. PCR reaction mixture

Ingredients	Quantity	Total volume
Genomic DNA	5µl (30µg/µl)	
Forward Primer	1µl	
Reverse Primer	1µl	25µl
DreamTaq Green PCR Master Mix (2X)	12.5 µl	
Injection water	5.5 µl	

***Antibiotic susceptibility assay*:** An antimicrobial susceptibility test was performed through the disk diffusion method. All isolates were tested against commonly used antibiotics; amoxicillin (AML) 25µg, ceftazidime (CAZ) 30µg, ciprofloxacin (CIP) 5µg, tetracycline (TE) 30µg, erythromycin (E) 15µg, and streptomycin (S) 10µg (Oxoid, UK). The disc diffusion method consisted of Inoculum preparation (0.5 Mc-Farland Standard), preparation of bacteria lawn on Muller Hinton agar (MHA) (Oxoid, UK), disc placement and zone measurement. Diverse antibiotic discs were positioned on MHA plates and incubated for 24h at 37ºC. The inhibition zone was calculated to the closest millimeter by using the Vernier Caliper and the results were noted according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

***Phenotypic detection of extended-spectrum beta -lactamases (ESBLs)*:** The Double Disk Synergy Test (DDST) was used to detect the presence of ESBLs. Bacterial lawn was applied onto MHA plates and exposed to ceftriaxone (CRO, 30µg), ceftazidime (CAZ, 30µg), cefotaxime (CTX, 30µg), aztreonam (ATM, 30µg), and amoxicillin/clavulanic acid (20/10µg) (Oxoid, UK), at a distance of 20mm from the center amoxicillin/clavulanic acid (20/10µg) disk to observe any synergistic effects (Kaur *et al*., 2013).

***Molecular detection of extended-spectrum beta-lactamases (ESBLs)*:** Molecular detection of the ESBLs, was done with PCR using ^{bla}*SHV*, *TEM*, and *CTX-M* genes. The primers sequence, PCR amplification conditions and reaction, are listed in Tables 1 and 2. PCR amplification was performed in a thermal cycler (Model 1861096, Bio-Rad, USA). The PCR products of 3µl were electrophoresed on 1% agarose stained with 7µl ethidium bromide and visualized under a Gel Documentation System (Bio-Rad, USA) (Monstein *et al*., 2017).

***Sequencing and phylogenetic analysis*:** Sanger sequencing (BGI, Hong Kong Co. Ltd., China) was performed to sequence the identified genes, including *16S rRNA*, *Usp*A, *vtx1*, *ipaH*, *aggR*, *est*, ^{bla}*SHV*, *TEM* and *CTX-M* using forward primers as the sequencing primers. The obtained sequencing data was submitted to NCBI GenBank for the accession numbers. The sequences were aligned with those from NCBI using Bio-Edit and ClustalW (Hall, 2011), and a Neighbor-Joining tree of the *16S rRNA* and *Usp*A genes were constructed using MEGA 11 software (Tamura *et al*., 2013).

***Statistical analysis*:** Descriptive statistics, frequency table, was used to interpret the percentage of the prevalence of *E. coli*, DEC pathotypes, virulence, and ESBLs genes. Furthermore, Chi-square test of independence was performed to compare the prevalence of *E. coli*, DEC pathotypes, virulence, and ESBLs genes at a *P*-value (≤ 0.05) (Kibret and Abera, 2011)

RESULTS

Clinical and postmortem examination and isolation of E. coli: A total of 516 (300 healthy and 216 diseased fish) fish samples were examined clinically and postmortem, revealing the presence of infection. In healthy fish, no abnormalities were seen externally, *i.e*., no hemorrhage, no skin lesions, no exophthalmia, no congested gills, fins, vent, normal abdomen, and anus. Internal examination revealed normal liver, intestine, and kidney.

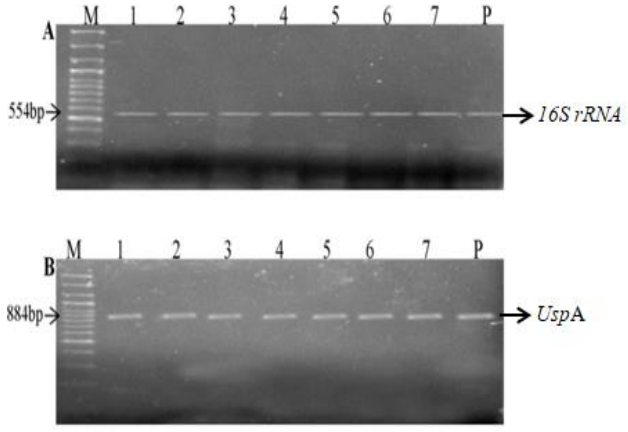

Figure 2. Agarose gel electrophoresis of PCR product for detection of *16S rRNA* (554bp) and *Usp*A (884bp) gene. (M) Shows molecular weight marker of Gene Ruler 100bp (Thermo-Scientific). (A) *16S rRNA* (554bp) Lane 1-4 healthy fish isolates, Lane 5-7 diseased fish isolates. (B) *Usp*A (884bp) Lane 1-4 healthy fish and Lane 5-7 denoted diseased fish isolates, and Lane P denoted positive control.

On the other hand, the above symptoms were observed in diseased fish samples. All fish samples were screened for *E. coli* using differential media (MacConkey) and selective Eosin Methylene Blue (EMB) to identify and differentiate *E. coli* from other bacterial species. Overall, 50/300 (16.66%) and 79/216 (36.57%) *E. coli* strains from healthy and diseased fish, respectively, resulted in pink color colonies on MacConkey agar and metallic green sheen with a dark central spot appearance on EMB agar confirming the *E. coli*.

***Biochemical test and molecular confirmation of E. coli*:** A total of 50/300 (16.66%) and 79/216 (36.57%) isolates were further confirmed with biochemical tests and PCR using *16S rRNA* and *Usp*A genes. In Figure 2, the bands recognized the *16S rRNA* (554bp) and *Usp*A (884bp) genes in *E. coli*.

***Prevalence of E. coli in farmed raised healthy and diseased fish*:** No statistical significance was found among the prevalence data. The data were not fit for the Chi-square and Fisher's exact test. However, no direction has been shown by Chi-square and Fisher's exact test in Table 3.

Table 3. Results of statistical analysis; chi-square test of independence showing X^2-value and P-value with respect to the selected parameters

Parameters	X^2-value	P-value
Fish Species to Fish Nature	10.0	0.440^{ns}
Fish Species to Fish Species	54.0	0.324^{ns}
Sampling Sites to Fish Nature	8.0	0.534^{ns}
Sampling Sites to Sites	48.0	0.352^{ns}

^{ns} = Shows non-significant result

To overcome these frequency analyses were used for further exploration. The prevalence of *E. coli* was categorized by the sampling area and fish species. Overall prevalence of *E. coli* was observed 16.66% (50/300) and 36.57% (79/216) of healthy and diseased farmed fish. With respect to the sampling area, a numerically a higher rate of *E. coli* was recorded in Multan 28% (14/50) and 50% (18/36), while a low prevalence 6% (3/50) and 22.22 % (8/36) was observed in Kasur in Table 4. On the other hand, the presence of bacteria with respect to species were studied in healthy and diseased fish; a higher frequency of isolates was documented in *C. carpio* 26% (13/50) and 47.22% (17/36), followed by *H. molitrix* 22% (11/50) and 41.66% (15/36) respectively, and a lower rate of prevalence was examined in *L. rohita* 8% (4/50) and 27.65% (10/36), respectively, in Table 4.

***Diarrheagenic Escherichia coli from healthy and diseased farmed fish*:** The isolates from healthy and diseased farmed fish carried virulent genes (*aggR*, *ipaH*, *vtx1*, and *est*) of different frequencies and a significant $P \leq 0.05$ were observed in Table 5. In Figure 3, the bands recognized the virulent genes (*aggR*, *ipaH*, *vtx1*, and *est*).

Table 4. Prevalence of *E. coli* with respect to species and sampling area

Prevalence of *E. coli* with respect to Species							
Fish	***L. rohita* (n= 50 N=36)**	***C. catla* (n= 50 N=36)**	***C. mrigala* (n= 50 N=36)**	***C. idella* (n= 50 N=36)**	***H. molitrix* (n= 50 N=36)**	***C. carpio* (n= 50 N=36)**	***E. coli* Isolates (n=50/300) (N=79/216)**
Healthy fish	4 (8%)	6 (12%)	9 (18%)	7 (14%)	11(22%)	13(26%)	50/300(16.66%)
Diseased fish	10 (27.65%)	12 (33.33%)	14 (38.88%)	11 (30.55%)	15 (41.66%)	17(47.22%)	79/216(36.57%)
Prevalence of *E. coli* with respect to Area							
Fish	**Muzaffargarh (n= 50 N=36)**	**Gujranwala (n= 50 N=36)**	**Bahawalnagar (n= 50 N=36)**	**Multan (n= 50 N=36)**	**Toba Tek Singh (n= 50 N=36)**	**Kasur (n= 50 N=36)**	***E. coli* Isolates (n=129)**
Healthy fish	12(24%)	5 (10%)	9 (18%)	14 (28%)	7 (14%)	3 (6%)	50/300(16.66%)
Diseased fish	16 (44.44%)	11 (30.55%)	14 (38.88%)	18 (50%)	12 (33.33%)	8 (22.22 %)	79/216(36.57%)

*Total 300 healthy farmed fish (n=50 of each fish species), and 216 diseased farmed fish (N=36 of each fish species)

Table 5. Prevalence of virulence genes among *E. coli* from healthy and diseased fish

Pathogenic *E. coli*	Genes	*E. coli* from healthy fish (n=50)	*E. coli* diseased fish (n=79)	DEC (n=64)	P- value
EAEC	*aggR*	8 (16%)	17(21.51%)	25 (39.06%)	<0.05
EIEC	*IpaH*	0	13(16.45%)	13 (20.31%)	<0.05
ETEC	*Est*	4(8%)	8 (10.12%)	12(18.75%)	<0.05
VTEC	*vtx1*	3(6%)	11(13.92%)	14(21.87%)	<0.05

Table 6. Distributions of non-pathogenic and pathogenic *E. coli* strains in farm and market fish

E. coli types	Healthy fish Isolates (n=50)	Infected Fish Isolates (n=79)
DEC isolates	15 (30%)	49(62.02%)
Non-DEC isolates	35 (70%)	30 (37.97%)

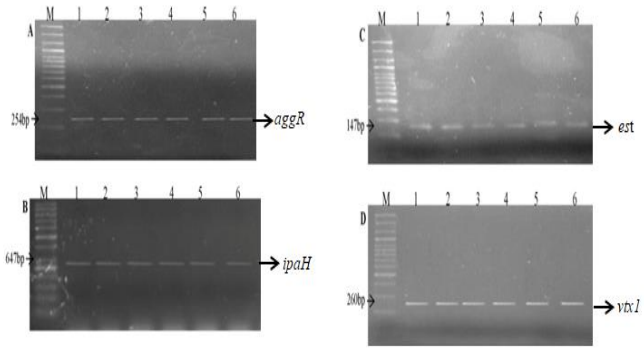

Figure 3. Agarose gel electrophoresis of PCR product for detection of *aggR, ipaH, est*, and *vtx1* gene. (M) Shows molecular weight marker of Gene Ruler 100bp (Thermo-Scientific). (A) Lane 1-6 represents *aggR* (254bp). (B) Lane 1-6 represents *ipaH* (647bp) gene. (C) Lane 1-6 represents *est* (147bp) gene. (D) Lane 1-6 represents *vtx1* (260bp) gene.

In general, a higher rate of *aggR* in 21.51% (17/79) was recorded in diseased fish isolates and 16% (8/50) of isolates from healthy fish, followed by *est* gene in 8% (4/50) and 10.12% (8/79) of isolates from healthy and diseased fish, respectively. The existence of *vtx1* gene in *E. coli* was documented in 6% (3/50) isolates from healthy and 13.92% (11/79) in diseased fish isolates. The *IpaH* gene was detected only in 16.45% (13/79) of isolates from diseased fish. While *IpaH* gene was not detected in healthy fish isolates in Table 5. Approximately 49.61% (64/129) isolates were confirmed DEC pathotypes; In general, 39.06% (25/64) *E. coli* isolates were from EAEC pathogroup, 18.75% (12/64) belonged to ETEC, and 21.87% (14/64) isolates were VTEC from both healthy and diseased fish, while 20.31% (13/64) of EIEC pathogroup was only observed in diseased fish isolates Table 5.

Based on the investigation and distribution of various virulence genes, the isolates were categorized into pathogenic and non-pathogenic groups in Table 6.

The prevalence of DEC regarding fish species both in healthy and diseased fish, a higher rate 40% (6/15) and 36.73% (18/49) was observed in *C. carpio*, followed by *H. molitrix* 26.66% (4/15) and 20.40% (10/49) presented in Table 7.

***Antimicrobial susceptibility*:** The highest resistance rates among diarrheagenic *E. coli* and non- diarrheagenic strains were observed against tetracycline, amoxicillin, and ceftazidime. In general, EAEC showed a higher resistance 84% (21/25) against tetracycline, followed by amoxicillin 72% (18/25), ceftazidime 64% (16/25) and 100% (25/25) sensitivity against ciprofloxacin, while EIEC strains carried a resistance against tetracycline 69.23% (9/13), amoxicillin

Table 7. Distribution of DEC pathotypes among the healthy and diseased farmed fish

Healthy fish	DEC pathotypes				Total isolates (n=15)	P-value
	EAEC (n=8)	EIEC (n=0)	ETEC (n=4)	VTEC (n=3)		
L. rohita	0	0	0	0	0	
C. catla	0	0	1(25%)	0	1(6.66%)	0.261
C. mrigala	2 (25%)	0	1(25%)	0	3 (20%)	0.238
C. idella	1(12.5%)	0	0	0	1(6.66%)	0.261
H. molitrix	2 (25%)	0	1(25%)	1(33.33%)	4 (26.66%)	0.238
C. carpio	3 (37.5%)	0	1(25%)	2(66.66%)	6 (40%)	0.213
	DEC pathotypes					
Diseased fish	**EAEC (n=17)**	**EIEC (n=13)**	**ETEC(n=8)**	**VTEC (n=11)**	**Total isolates (n=49)**	**P-value**
L. rohita	1 (5.88%)	2 (15.38%)	0	1(9.09%)	4 (8.16%)	0.238
C. catla	3 (17.64%)	1 (7.69%)	1 (12.5%)	1(9.09%)	6 (12.24%)	0.261
C. mrigala	2 (11.76%)	1 (7.69%)	2(25%)	1(9.09%)	6 (12.24%	0.261
C. idella	1 (5.88%)	2 (15.38%)	1 (12.5)	1(9.09%)	5 (10.20%)	0.261
H. molitrix	4 (23.52%)	3 (23.07%)	1 (12.5%)	2 (18.18%)	10 (20.40%)	0.213
C. carpio	6 (35.29%)	4 (30.76%)	3 (37.5%)	5 (45.45%)	18 (36.73)	0.213

Table 8. Results of antimicrobial susceptibility

Antibiotics	EAEC (n=25)			EIEC (n=13)			ETEC(n=12)			VTEC(n=14)			Non-DEC(n=65)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
AML	18 (72%)	2 (8%)	5 (20%)	8 (61%)	1 (8%)	4 (31%)	12 (100%)	0	0	9 (64%)	2 (14%)	3 (21%)	44 (68%)	3 (5%)	17 (26%)
CAZ	16 (64%)	3 (12%)	6 (24%)	6 (46%)	4 (31%)	3 (23%)	9 (75%)	1 (8%)	2 (17%)	7 (50%)	1 (7%)	6 (43%)	37 (57%)	8 (12%)	22 (34%)
CIP	0	0	25 (100%)	0	0	13 (100%)	0	0	12 (100%)	2 (14%)	0	12 (86%)	0	0	65 (100%)
TE	21 (84%)	1 (4%)	2 (8%)	9 (69%)	2 (15%)	2 (15%)	12 (100%)	0	0	13 (93%)	0	1 (7%)	46 (71%)	5 (8%)	14 (21%)
S	13 (52%)	4 (16%)	8 (32%)	5 (38%)	1 (8%)	7 (54%)	4 (33%)	1 (8%)	7 (58%)	3 (21%)	1 (7%)	9 (64%)	32 (49%)	11 (17%)	22 (34%)
E	9 (36%)	5 (20%)	11 (44%)	3 (23%)	1 (8%)	9 (69%)	2 (17%)	1 (8%)	9 (75%)	2 (14%)	0	11 (8%)	19 (29%)	7 (11%)	39 (60%)

Amoxicillin (AML), Ceftazidime (CAZ), Ciprofloxacin (CIP), Tetracycline (TE), Streptomycin (S), Erythromycin (E)

Table 9. Genotypic detection of ESBLs among DEC pathotypes

E. coli types	DEC (n=64)	ESBLs genes			Total ESBLs positive isolates (n=44)	P-value
		TEM (n=19)	^{bla}*SHV*	*CTX-M* (n=25)		
EAEC	25 (39.06%)	8/25 (32%)	0	11/25 (44%)	19/44 (43.18)	0.199
EIEC	13 (20.31%)	2/13(15.38%)	0	4/13(30.76%)	6/44(13.63%)	0.199
ETEC	12 (18.75%)	4/12(33.33%)	0	6/12(50%)	10/44(22.72%)	0.199
VTEC	14 (21.87%)	5/14(35.71%)	0	4/14(28.75%)	9/44(20.45%)	0.199

61.53% (8/13), ceftazidime 46.15% (6/13) and showed 100% (13/13) sensitivity against ciprofloxacin, on other hand, ETEC showed 100% resistance against tetracycline and amoxicillin and 100% sensitivity against ciprofloxacin. The resistance of VTEC strains against tetracycline and amoxicillin were recorded in 92.85% (13/14) and 64.28% (9/14) of isolates respectively, while 85.71% (12/14) of isolates showed sensitivity to ciprofloxacin. Furthermore, non-DEC strains also showed a higher resistance against tetracycline 70.76% (46/65) and amoxicillin 67.69% (44/65), while all isolates were ciprofloxacin susceptible. The antimicrobial susceptibility results of both DEC and non-DEC strains obtained from healthy and diseased farmed fish are presented in Table 8.

***Extended-spectrum beta-lactamase identification among dec and non-dec pathotypes*:** Out of 64 DEC isolates, ESBLs production was detected phenotypically in 68.75% (44/64) of isolates Figure 4. However, ESBL genes were examined using PCR in Figure 5, isolates were found to harbor *CTX-M* and *TEM* genes, as 56.81% (25/44) were found positive for *CTX -M* gene, while 43.18% (19/44) were for *TEM* gene in Table 9. Co-occurrence of both genes was observed in 8/44

(18.18%) of the isolates, but none of them carried the ^{bla}*SHV* gene. On the other hand non-DEC isolates were also ESBLs positive. Phenotypically 18.46% (12/65) isolates were recorded ESBLs positive, as 18.46% (12/65) of isolates harbored the 41.66% (5/12) *TEM* and 58.33% (7/12) *CTX-M* genes, while ^{bla}*SHV* gene was not detected in any of the isolates. No statistical significance was found among the prevalence of ESBL genes.

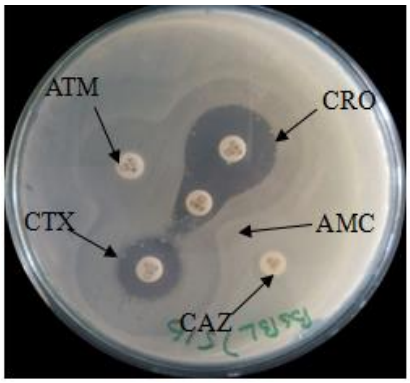

Figure 4. Results of phenotypic detection of ESBLs. CRO shows the synergetic effect to AMC.

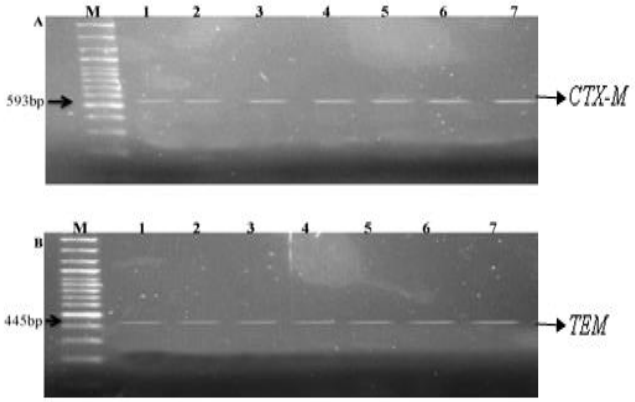

Figure 5. Agarose gel electrophoresis of PCR product for detection of *CTX-M* and *TEM* genes. (M) Shows molecular weight marker of Gene Ruler 100bp (Thermo-Scientific). (A) Lane 1-7 represents *CTX-M* (593bp). (B) Lane 1-7 represents *TEM* (445bp).

Sequence and phylogenetic analysis: PCR products of *16s rRNA* and *Usp*A, *vtx1*, *ipaH*, *aggR*, *est*, *TEM*, and *CTX-M* genes were sequenced. Nucleotide sequences were blasted and aligned with NCBI GenBank data to get the accession number in Table 10 and compared the nucleotide similarity potential of *E. coli* strain with other exiting in the database, 95-100% of similarity has been shown. Furthermore, the *16S rRNA* and *Usp*A gene sequenced data were analyzed with phylogeny (Neighbor-Joining tree) characterization in Figure 6 and 7 respectively.

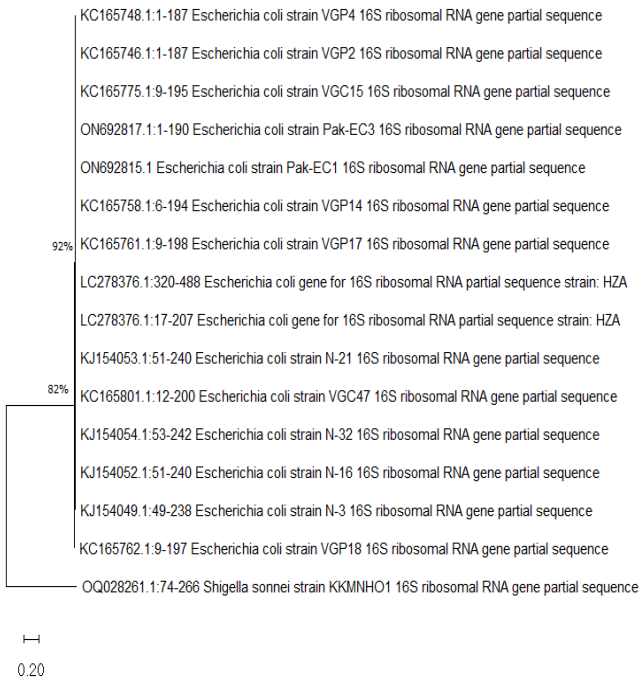

Figure 6. Neighbor-Joining tree using 16S rRNA gene. The 92 % similarity has been recorded with database sequences.

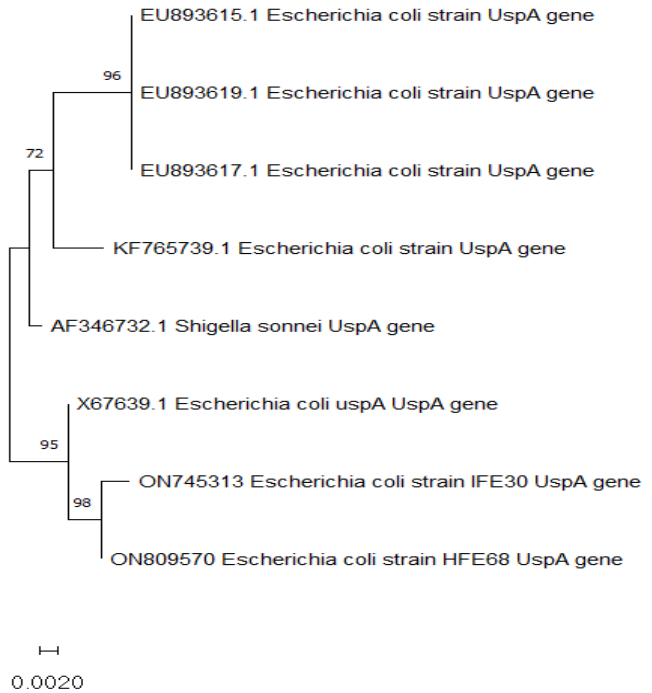

Figure 7. Neighbor-Joining tree using *Usp*A gene. The 96% similarity has been recorded with database sequences.

Table 10. NCBI accession number of detected genes

Antibiotic	Gene	Strain Name	NCBI acession no.
Universal Stress Proteins	*Usp*A	HFE68	ON809570
		IFE30	ON745313
16S r RNA	*16S r RNA*	Pak-EC3	ON692817
		Pak-EC1	ON692815
Enteroaggregative *E. coli* (EAEC)	*aggR*	IFE40	ON950428
		HFE53	ON950430
Enterotoxigenic *E. coli* (ETEC)	*Est*	IFE21	ON968441
		HFE8	ON968443
Verocytotoxin-producing *E. coli* (VTEC)	*vtx*1	IFE44	ON968438
		HFE12	ON968440
Enteroinvasive *E. coli* (EIEC)	*IpaH*	IFE3	OP114848
ESBLs	*CTX-M*	HFE4	OP156933
	TEM	HFE7	OP227117

DISCUSSION

E. coli presence and their DEC pathogroups in fish species from different fish farms were observed in the present study. The incidence of *E. coli* and their pathogroups have been evaluated worldwide in different animal species like; fish, birds, amphibians, mammals, invertebrates, livestock, seafood, and aquatic environment (Persad *et al*., 2015; Espinosa *et al*., 2018). Furthermore, our findings were compared with available data. The presence of *E. coli* was investigated in faecal samples of fish; a total 81.51% prevalence was reported from different geographical regions of Rajasthan, India (Saharan *et al*., 2020). Another investigation from Bangladesh, has recorded *E. coli* contamination in fish (75.6%), baskets (68.8%), and mat (75%) from fish farms and fish markets (Ava *et al*., 2020). *E. coli* in Indian major carps (65%) and 85% in shrimps from domestic fish markets have been in Kolkata and West Bengal, India (Dutta and Sengupta, 2016)

In the current study, *E. coli* presence was noticed in various fish species. A previous study in Turkey, 60 % of *E. coli* from cage culture *O. mykiss* (Onmaz *et al*., 2020) and 12% of *E. coli* from tissue of *C. gariepinus* and *O. niloticus* has been reported (Haile and Getahun, 2018). In freshwater polycultured carps in India, the author reported 19.4% in *C. catla*, 35.34% in *L. rohita*, 21.42% in *C. mrigala* and 29.64% in *C. carpio* (Saswathi *et al*., 2015). The prevalence of *E. coli* has been documented in livestock integrated fish farms; a higher rate has been noted in the gut and muscle of *C. idella*, *H. molitrix*, and *L. rohita* (Dang and Dalsgaard, 2012)

E. coli isolates were further categorized into distinct pathogroups, in which four different DEC pathotypes have been reported. The findings derived from this study indicate that healthy and diseased fish from different fish farms may exhibit DEC pathotypes. There has been limited data available for DEC strains in farm raised healthy and diseased fish and various methods have been employed by researchers in different studies. The prevalence in finfish and shellfish; enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC and enteroaggregative *E. coli* (EAEC) has been tested with PCR using different genes (Prakasan *et al*., 2022). Few previous reports in West Africa pathotypes of *E. coli* from farm *O. niloticus* were screened with different genes; with a percent prevalence of 8.33% Shiga toxigenic *E. coli* (STEC), 3.33% EIEC and 31% EHEC and 28% *Shigella* was isolated from market fish in Houston, Texas, USA (Okyer *et al*., 2018; Kouadio-Ngbesso *et al*., 2019). In a previous study, the pathogenic *E. coli* was revealed in *O. niloticus* and water with different virulence genes in Egypt (Galal *et al*., 2013). The presence of virulence genes and pathotypes; ETEC, EPEC, and EAEC, has been studied in marine sediments (Vignaroli *et al*., 2012). The survival of pathogenic *E. coli* strains has been investigated with different virulence genes in an aquatic environment contaminated with distinct sources (Berthe *et al*., 2013). Another study resulted STEC, EPEC, EIEC, EAEC, and ETEC in fish and their products (Koo *et al*., 2012). Similarly, a study was performed in Tunisia; they have been documented 53.3% of ETEC, 16.6% of EAEC, and 6.6% of EIEC in wastewater (Salem *et al*., 2011).

The findings of this study indicate a remarkably high prevalence of antibiotic resistance among *E. coli* isolates. The figures presented in this study were compared with the local data published by Shah *et al*. (2012) and Ahmad *et al*. (2022) who worked on isolated bacteria from farm water, sediments, diseased and market fish. In their published data they reported the resistance profile of tetracycline 43.3%, streptomycin 36.2%, amoxicillin 71.6%, and ESBLs 16% against the isolated bacteria and these results were almost in line with the confirmation of the present study. Our results are consistent with previous research studies that reported *E. coli* isolates from children in Egypt and Iran, having high frequencies of antibiotic susceptibility, as well as the presence of various resistance genes such as ^{bla}*TEM* (78.9%), (36.3%), and ^{bla}*CTX-M* (78.9%), (22.7%), respectively (Khoshvaght *et al*., 2014; Khairy *et al*., 2020). A previous reported that 78% of both DEC and non-DEC isolates carried ESBLs genes (Ali *et al*., 2014), while another study highlighted an even higher percentage of positive ESBLs genes at 93.3% (Zhou *et al*., 2018). Similarly, another prior study evaluated three different aquaculture farms in China to screen *E. coli* isolates; the analysis reported ^{bla}*CTX−M* (80%) and ^{bla}*TEM* (60%) isolated from farm I water and sediments, in farm II ^{bla}*CTX−M* (70%), and ^{bla}*TEM* (60%), farm III ^{bla}*CTX−M* (80%), and ^{bla}*TEM* (80%), were reported in water isolates (Liao *et al*., 2021). In Egypt, an experiment documented ^{bla}*CTX-M-15* and ^{bla}*TEM* in *E. coli* from integrated fish farms. The cited studies concluded that *E. coli* isolates from different sources (farm, fish, and fishery products) carried the ESBLs genes, and

similar findings were observed in the current investigation (Hamza *et al*., 2020).

Multiple factors may be responsible for the variations in the prevalence rates of DEC pathotypes and antibiotic resistance and resistant traits observed in the current research and previous research studies, including geographical location, study participants, and sanitation standards. It's important to acknowledge that these factors are not comprehensive and that there may be other possible reasons for the differences.

***Conclusion*:** The findings of the present study indicate that unsanitary practices in aquaculture and aquatic environments may not only impact farmed fish, but also pose a risk to public health. Therefore, it is necessary to implement strict regulations to monitor and prevent unhygienic practices in aquaculture and associated environments.

***Authors' Contribution Statements*:** MU: Execution of the experiment and collection of the data, writing the manuscript. FR: Conceiving and designing the study. NK: Help in sampling. SA: Assistance in statistical analysis. SAA: Assistance in experimentation.

***Conflict of interest*:** The authors declare that they have no known financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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