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Prevalence of *Escherichia coli* Resistance Genes in Mullet Fish

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Abstract

Escherichia coli gene mutations (plasmids, integrons, and transposons) have instigated multidrug resistance (MDR) against various antimicrobials. This study detected antibiotic-resistance genes (genotypic and phenotypic) in *E. coli* and performed Whole-genome sequencing to discover MDR-associated *E. coli* genes in mullet fish. *E. coli* isolates were serologically identified and their antimicrobial sensitivity was tested. *E. coli* presence was confirmed in 35% of the mullet fish samples. Cephalosporins, tetracyclines, and sulfonamides presented higher efficiency against *E. coli*. The serological investigation revealed the presence of STEC (O157:H7, 50%), ETEC (O142, 20%), EHEC (O26:H11,15%), and EPEC (15%) in mullet meat samples. A symmetrical band represented the Subunit B of the Shiga-like toxin (SLT) genewhereas the Heat-labile toxin (LT) gene was found in plasmid and genomic DNA-detected strains. The results revealed the hazardous nature of STEC for mullet meat consumers. The study recommends improving the hygienic conditions during the mullet handling and processing steps, which will minimize antibiotic usage and resistance.

KEYWORDS
Integrons, Shiga toxin-producing *E. coli*, Gentamycin, Heat-Labile Toxin, plasmids.

INTRODUCTION

Escherichia coli is a gram-negative nonpathogenic commensal microorganism of human and animal intestines. They are mainly classified as Enterohaemorrhagic, Enteropathogenic, Enterotoxigenic, and Enteroinvasive. However, infectious Shiga toxin-producing *E. coli* (STEC) can cause various gastrointestinal disorders such as hemolytic-uraemic syndrome (HUS), hemorrhagic colitis (HC), and diarrhea through the consumption of contaminated food and water (Adeyanju and Ishola, 2014).

The flathead grey mullet, scientifically named Mugil cephalus, is a prominent member of the Mugilidae family and holds considerable significance as a food fish. These mullets, commonly known as grey mullets, constitute a group of ray-finned fish distributed in coastal temperate and tropical waters worldwide, with some species even thriving in freshwater environments. Beyond their crucial role in fisheries and aquaculture, the flathead grey mullet serves as a representative species within this fish family. Typically, they range in size from 15 to 30 centimeters and go by various English names, including flathead common mullet, striped mullet, black mullet, bully mullet, sea mullet, grey mullet, and simply mullet, among others. Adult flathead grey mullets primarily feed on algae in freshwater habitats. One remarkable feature of this species is their euryhaline nature, which means they possess the ability to adapt to changing levels of salinity in their environment. This adaptability equips them to thrive equally well in both freshwater and saltwater environments, making them exceptionally versatile and resilient within various aquatic ecosystems (Whitfield *et al*., 2012; Crosetti, 2015).

Mullets have served as an important source of food in Mediterranean Europe since Roman times. The family includes about 78 species in 20 genera. Mullet fish, contaminated food products, and water are the primary source of pathogenic STEC infection in humans. The fecal matters could contaminate the meats (mutton, mulletmullet, and beef) with STEC during slaughtering, handling, processing, and storage (Victoria *et al*., 2022).

Antibiotic treatments are the main remedy to counter *E. coli* infections, which have led to the emergence of multidrug-resistant (MDR) *E. coli* strains. The antibacterial resistance of microorganisms is mainly attributed to gene mutation, and resistance genes found in integrons, transposons, and plasmids.

The activation cassette-containing integrons are associted with gene expressions. Two conversational segments (CS) are found in Integron Class I adjacent to the variable region (VR), which are common in the new generation of Enterobacteriaceae (CDC, 2019). The overuse of antimicrobials leads to the emergence of a new generation of mutated microbial strains with higher antibacterial resistance. This scenario is considered a serious global public health concern. Therefore, novel drugs are being investigated to treat MDR strains in poultry, animals, and humans (Cunrath, *et al*., 2019).

This study aimed to explore the antimicrobial resistance (genotypic and phenotypic) and Whole-genome sequencing based identification of MDR-related gene structure of *E. coli* in commercial mullet meat.

MATERIALS AND METHODS

Sampling, preparation, and bacterial Testing

A total of 100 mullet samples were chosen at random from different marketplaces. The samples were put in polyethylene

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bags and moved right away to the bacteriological lab's refrigerator for analysis. A 2g sample of homogenized mullet meat was cultured in MacConkey broth after an 18-hour incubation period at 37°C. It was then streaked onto an Oxoid MacConkey Agar Medium plate and incubated for 24 hours at 37°C. The pink colonies were painted on Oxoid and incubated at 37°C for 24 hours. The enormous *E. coli* colonies had a metallic sheen that was blue, black, and green. Biochemical test kits (bioMerieux API, France) and morphological and microscopic studies were used to identify *E. coli* colonies (CDC, 2020). Additionally, the WHO-recommended antiserum set (Denka Seiken Co., Japan) was used for serotyping (Ewing, 1986).

Antibacterial susceptibility

Disc diffusion method was adopted to assess antibacterial susceptibility using Mueller-Hinton agar and 12 antibiotic discs (Tetracycline, Gentamycin, Ciprofloxacin, Streptomycin, Trimethophrim, Cefepim, Doxycycline, Cefotaxim, Flumequine, , Sulfamethoxazole, Penicillin, and Ampicillin). The concentrations of Penicillin and Ampicillin were 10 µg/disc whereas the concentrations of other discs were 30 µg/disc (Alderman and Smith, 2001).

Serological identification of E. coli using slide agglutination test

Monovalent and polyvalent standard anti-selenium revealed the tested microorganism as enteric pathogenic *E. coli* (EPEC) (Li *et al*., 2013). The microbial colonies were emulsified by adding two saline drops to the glass slide. To test the monovalent serum, nutrient gradient agar was used to cultivate another colony (37°C and 24 hours) with the addition and aggregation of looped antiserum. Microorganisms' suspension was prepared in physiological saline and antigen was identified through a slide agglutination test.

Molecular Identification

DNA extraction

GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, USA) was used for DNA extraction. Briefly, microbial colonies were centrifuged (5000 µg for 10 minutes), and the cell pellet was resuspended in 180 µl solution for digestion. Then, K Proteinase was added (20 µl), mixed, and bathed in water followed by incubation for 30 minutes at 56°C with continuous shaking for complete dissolution. RNase solution (20 µl) was added to the mixture, incubated (10 minutes at 37°C), and vortexed followed by the addition of ethanol (50%, 400 µl) and vortexing. The lysed cells were transferred, purified, and centrifuged at 6000 µg for 1 minute. Washing buffer (I & II) (500 µL) was added to the column and centrifuged for 2 minutes at the maximum speed for the complete removal of ethanol. The purified DNA was stored at -20°C. Plasmid preparation was carried out using a DNA plasmid GeneJet miniprep kit (Thermofisher Scientific, USA). Microbial colonies were placed in a micro-centrifuge tube (1.5 ml) and centrifuged (12,000 xg /2 min). The ice-cold buffer solution (250 µl) was used for pellet resuspension. The tube was inverted 56 times for proper mixing and incubated for 5 minutes at 37°C. The supernatant was transferred and centrifuged for 30 seconds at 10,000 xg. Buffer (500 µl) was used for rinsing followed by centrifugation at 10,000 xg for 30 seconds. Preheated ddH_2O (50 µl) was used to elute the DNA, which was incubated at 37°C for 3 minutes. Then, centrifugation was carried out at maximum speed (14,000xg) for 30 seconds.

Gene amplification

The PCR reaction mixture (25 µl) consisted of purified genetic material (1 µl, genomic DNA / plasmid prep), MgCl2 (2.5 µl), buffer (5 µl), primer (1 µl, Table 1), Taq polymerase (0.25 µl), dNTP (0.5 µl), and nuclease-free water. Agarose gel (1%) containing ethidium bromide (0.5 µg / ml) was used for the gel electrophoresis (80V for 50 minutes) of PCR products. DNA ladder of 100 bp was used to compare the target DNA size and imaged under a Gel documentation system (Biometra, Göttingen, Germany).

Extraction of DNA fragments

DNA Extraction Kit (Thermofisher Scientific, USA) was used to extract the target DNA fragments from agarose gel under UV light and stored in a 1.5 ml tube. The tube containing extracted DNA fragment was centrifuged for 2 minutes at 13000 xg. Buffer (700 µl) was used to wash the column followed by centrifugation for 1 minute at 37°C. The buffer (50 µl) was again added to elute with a spin column filter, held for 1 minute at 37°C, and centrifuged for 2 minutes at 13,000 x g.

Statistical analysis

SPSS software was used to perform ANOVA to compare the means.

RESULTS

E. coli Prevalence

The results revealed *E. coli* prevalence in 35% of mullet meat samples (Figure 1).

Antibacterial resistance pattern of E. coli

Antibacterial susceptibility of mullet meat-isolated *E. coli* strains (n = 20) against 12 antibiotics belonging to six different classes is presented in Table 2. Sulfonamides were the most ef-

Table 1. Primer sequences for target gene amplification.

Target Gene	Primer sequence
Shiga-like toxin (SLT)	SLT F:5'-AAGAAGATGTTTATGGCGGTTT-3' SLT R:3'-GTCATTATTAAACTGCACTTCAGCA-5'
Heat-labile toxin (LT)	LT F:5'-ATTGACATCATGTTGCATATAGGTTAG-3' LT R:3'-ACATTTTACTTTATTCATAATTCATCCCG-5'
Ciprofloxacin resistance gene	*aac (6')-F:5'-TTTATTATTTTTAAGCGTGCATAATAAGCC-3'* *aac (6')-R:3'-TTAAGACCCTTAATTGTTGGGATTT-5'*
Gentamycin resistance gene	*aac* C2-F:5'-CATACGCGGAAGGCAATAAC-3' *aac* C2-R:3'-ACCTGAAGGCTCGCAAGA-5'

fective antibiotics against isolated *E. coli* strains [trimethoprim (20/20) 100% and sulfamethoxazole (16/20) 80%]. The efficacy of cephalosporins such as Cefepime and Cefotaxime was noted as 70% (14/20) and 65% (13/20) against *E. coli* strains, respectively. Tetracyclines including Tetracycline and Doxycycline were effective against 50% (10/20) and 40% (8/20) *E. coli* strains, respectively. The other three antibiotic classes including Quinolones [ciprofloxacin (4/20) 20% and flumequine (4/20) 20%], aminoglycosides [gentamicin (3/20) 15% and streptomycin (2/20) 10%], and β-lactams [penicillin (2/20) 10%, and ampicillin (0/20) 0%] presented weak efficiency against isolated *E. coli* strains.

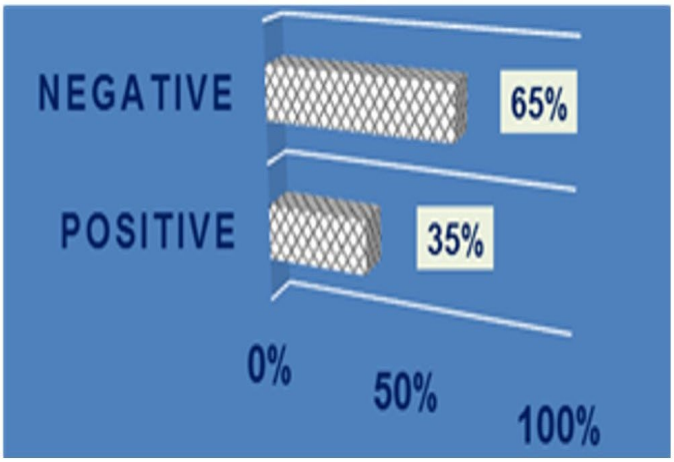

Figure 1. *E. coli* isolate frequency in mullet meat samples.

Serological identification of E. coli isolates

Serological testing identified 20 *E. coli* isolates in mullet meat samples (Figure 2). The presence of various *E. coli* isolates was noted as STEC [O157: H7,10/20 (50%)], ETEC [O142, 6/20 (30%)], EHEC [O26: H11, 2/20 (10%)], and EPEC [O55: H7, 2/20 (10%)]. Figure 3 reveals the screening of Shiga-like toxin (SLT). Subunit B of the SLT gene presented a uniform genomic DNA band of 300 bp. Results demonstrated minimal amplification of strain approximately 200 bp. A gentamicin resistance gene (*aac*C2) segment of 856 bp was found in both strains whereas a minor band was detected at 300 bp figure depicts a low prevalence of the Ciprofloxacin resistance gene in plasmid and genomic preparations.

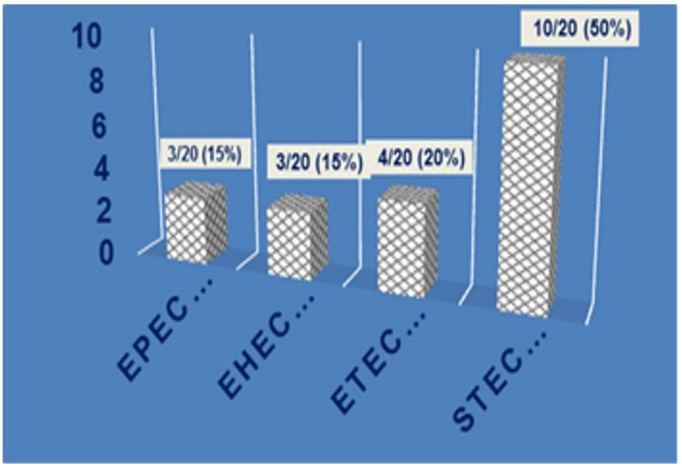

Figure 2. *E. coli* serotype prevalence in mullet flesh samples.

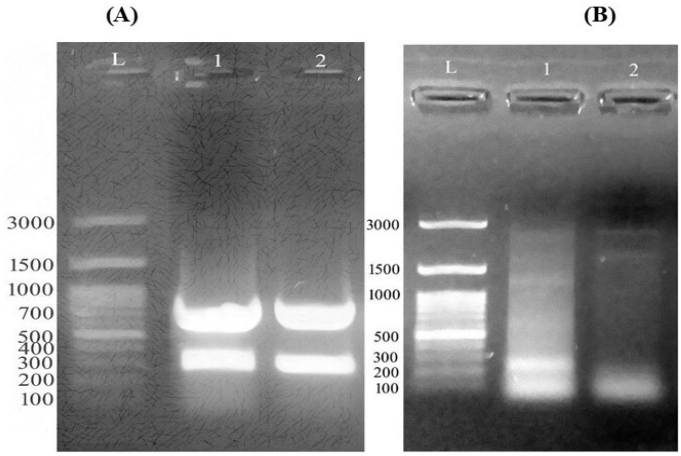

Figure 3. (A) 856 bp long gentamycin-resistant gene (*aac*C2). At 300 bp, a small band appeared. (B) 1 kb-long gene for ciprofloxacin resistance. Genomic amplification of strain.

DISCUSSION

The findings of this study are in accordance with Partridge *et al.* (2018) and Wu *et al.* (2018) who reported *E. coli* prevalence in 35.5% and 35.0% of tested mullet samples, respectively. The Indian mullet analysis revealed a 31% *E. coli* occurrence whereas *E. coli* presence in 20% of mullet samples has been reported in the US (Ngullie *et al.*, 2011; Sato *et al.*, 2010).

Shaltout et. al. (2020) reported *E. coli* isolation from 13.33% of Egyptian mullet meat samples whereas *E. coli* was noted in

Table 2. Antibiotic susceptibility of *E. coli* isolated from mullet meat samples.

Antibiotics	Antibacterial agents	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Sulfonamides	Trimethoprim	20	100	0	0	0	0
	Sulfamethoxazole	16	80	2	10	2	10
Cephalosporins	Cefepime	14	70	4	20	2	10
	Cefotaxime	13	65	4	20	3	15
Tetracycline	Tetracycline	10	50	0	0	10	50
	Doxycycline	8	40	2	10	10	50
Quinolones	Ciprofloxacin	4	20	4	20	12	60
	Flumequine	4	20	6	30	10	50
Aminoglycosides	Gentamicin	3	15	5	25	12	60
	Streptomycin	2	10	5	25	13	65
β-lactam	Penicillin	2	10	6	30	12	60
	Ampicillin	0	0	7	35	13	65

11.1 % of Nigerian mullets (Tomova *et al*., 2018). Liu *et al*. (2016) have demonstrated the presence of *E. coli* in 10.60% of Croatian mullet samples whereas Jakabi *et al*. (2002) observed a 9% occurrence of *E. coli* in mullet meat. Multiple studies have investigated Saudi Arabian mullet meat and revealed an *E. coli* prevalence of 5.92% (Deng *et al*., 2016; Schulz *et al*., 2015). The lowest *E. coli* occurrence (1.56%) has been reported in Moroccan mullet (Collins, 2000).

These reports indicates inadequate hygienic practices during slaughtering, handling, and transportation. The unhygienic meat processing could lead to *E. coli* presence in processed mullet meat indicating post-processing contamination (Collin, 2000). *E. coli* inhabits the gastrointestinal tracts of humans and animals. *E. coli* presence in prepared foods is associated with fecal contamination, which could also accompany other harmful microorganisms (*Salmonella*, *Campylobacter*, and *Shigella*) (Younis *et al*., 2017).

Antibacterial drugs prevent bacterial infections and serve as mullet growth promoters. The selection of a proper antibacterial agent is crucial for better output. The testing of antibacterial susceptibility against *E. coli* strains (n = 20) isolated from mullet meat samples revealed varying efficacy of different antibiotics such as sulfonamides [trimethoprim (20/20) 100%, sulfamethoxazole (16/20) 80%], cephalosporins [Cefepime (14/20) 70%, Cefotaxime (13/20) 65%], tetracyclines [Tetracycline (10/20) 50%, Doxycycline (8/20) 40%], Quinolones [ciprofloxacin (4/20) 20%, flumequine (4/20) 20%], aminoglycosides [gentamicin (3/20) 15%, streptomycin (2/20) 10%], and β-lactam [Penicillin (2/20) 10%, ampicillin (0/20)0%]. These results are in line with the recommendations of the CDC (2019).

Younis *et al*. (2017) have also reported almost complete *E. coli* resistance against penicillin, cefepime (95.8%), and amoxicillin (94.5%). Ammar *et al*. (2015) have associated *E. coli* antibiotic resistance with plasmid genes. Other studies have also demonstrated *E. coli* resistance (90%) against trimethoprim, ampicillin, cephalexin, gentamicin, tetracycline, sulfamethoxazole, and streptomycin (Bie *et al*., 2018; Adeyanju and Ishola, 2014).

Multidrug resistance of *E. coli* against sulfonamides, aminoglycosides, β-lactams, and tetracyclines is a well-known phenomenon (Ramadan *et al*., 2016). Different studies have reported the prevalence of β-lactam resistant *E. coli* (Mohamed *et al*., 2014; Eid and Erfan 2013).

Li *et al*. (2020) have reported high resistance of *E. coli* towards tetracycline, sulfadiazine, chloramphenicol, gentamicin, ceftriaxone, amoxicillin, sulfadiazine, and ampicillin. Zhang *et al*. (2012) have revealed that 60% of mullet meat-isolated *E. coli* were resistant to fluoroquinolones. Tang *et al*., (2011) have also reported considerable *E. coli* resistance to ciprofloxacin (35.0%), norfloxacin (36.8%), and enrofloxacin (34.1%).

STEC [O157: H7 (10/20) 50%], ETEC [O142 (6/20) 30%], EHEC [O26: H11 (2/20) 10%], and EPEC [O55: H7 (2/20) 10%] were all detected by serological testing of isolated *E. coli* strains (20). The *SLT* gene's subunit B displayed a consistent 300 bp-long DNA fragment. The findings showed that strain (1) amplified less than Heat Labile toxin (LT). In both strains (1 and 2), fragments of roughly 200 bp were observed.

The molecular size of the gentamicin resistance gene (*aac*C2) fragment was determined to be 856 bp, whereas a tiny band showed up at 300 bp. Additionally, both cultures' plasmid and genomic preparations were examined for ciprofloxacin-associated resistance genes (1 and 2). Strain (1) was the only one to have a 1 kb band. However, the tested strains' plasmid did not contain the target gene.

The findings of Momtaz and Jamshidi (2013), who discovered several O serotypes (O2, O35, O1, O8, O109, O18, O88, O15, O115, and O78), are consistent with these findings.From enteropathogenic *E. coli*, Ying *et al*. (2020) identified an *eae*A gene that resembled the eae genes of EHEC, O157: H7, and O55: H7. While HECO157 included a 60MDa plasmid, Kakoullis *et al*.'s (2019) detection of an *SLT* gene produced false negative results. Yang *et al*. (2020) have recorded the *Stx* gene in EHEC strains, whereas Lagerqvist *et al*. (2020) have reported the *eae*A and *SLT* (I, II) genes, which suggest the presence of EHEC O157 strain. Extraintestinal infectious genes such *tra*T, *afa*D8, *Eisen*, *Cdt2*, *bma*E, *cdt3*, *iut*A, and *iuc*D are among the virulence genes. According to Villegas *et al*. (2013), the ETEC strain contains the etpD gene.

RIMD 0509952 and EDL933 are known to cause intestinal hemorrhage whereas the fmH gene is considered a non-virulent gene in various *E. coli* strains (Momtaz and Jamshidi, 2013).

CONCLUSION

The study demonstrates *E. coli* pathogenicity-related genes in mullet meat samples, which included antigenic genes and different somatic capsules. STEC management is crucial as it could be hazardous to mullet meat consumers. The common presence of *E. coli* in daily meals could be harmful to food biosafety and public health. Therefore, enhanced hygiene measures should be adopted during the slaughtering, handling, and processing of mullet carcasses. Furthermore, unnecessary antibiotic administrations should also be avoided in humans and mullets to counter the emergence of new antibacterial resistance.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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