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Prevalence of Extended Spectrum B-Lactamse Producing, Antibiotic Resistant *Escherichia coli* in Fresh Fish and Fish Waste in Retail Fish Markets of Navi Mumbai, India

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ABSTRACT: Transmission of antimicrobial genes through chain remain major public threat globally. Antibiotic susceptibility patterns of *Escherichia coli* were studied on fresh seafood found in retail market. ESBL *E. coli* isolated from 430 (fresh fish, fish waste and associated environment) fresh fish samples were examined in light of the phenotypic traits associated with antibiotic resistance. Of them, 60 ESBL positive *E. coli* were isolated. A large number of isolates were amoxicillin-resistant (98.4%), cefepime (91.9%), and both cefepime and ceftazidime (91.1%). Relatively higher susceptibilities were recorded against colistin (100%) cefotaxime-clavulanic acid and amoxyclav each with 85.5% respectively. All 60 isolates showed resistance to more than 03 antibiotics. Two isolates EC01 and EC59 showed resistance to 14 antibiotics. MIC values of 87% isolates were 1024µg/ml of cefotaxime. E-test showed resistance against multi enzyme MIC™ strips for 20 isolates. The Multiple Antibiotic Resistance Index for the 60 isolates ranged from 0.2 to 0.87, showing that sources with a high risk of contamination had the highest levels of contamination. These studies reveal more prevalence of *E. coli* in fish waste samples than fresh fish. Thus, it draws attention to the threat of the spread of communities of seafood consumers who ingest multidrug resistant *E. coli* as well as the necessity of improving the sanitation of coastal waterways, retail markets, and landing areas. The present study gives direct contribution in monitoring antimicrobial gene in aquaculture value chain.

Keywords: Beta lactamase, *E. coli*, Seafood, MIC, Antibiotic Resistance.

INTRODUCTION

Microbial isolates with multidrug resistance (MDR) are those that are resistant to at least single agent from each of three antimicrobial groups (Magiorakos *et al.*, 2012). High protein diet has recently been given priority by low and middle-income countries and they are vulnerable to antibiotic resistance criteria due to limited surveillance and diagnostic procedures (Larsson & Flach 2022). Intensive farming has been facilitated with therapeutic use of antibiotics in animals and farming that contributes to increased antibiotic resistance (Van *et al.,* 2019). Antibiotics are used in various sectors have compounded risk of resistant bacteria being transferred to humans (Olaitan *et al*., 2015). Increase in antibiotic resistance to cephalosporin and carbapenem in most of the enterobacteriaceae family is a global concern. The only and last resort antibiotic is colistin against MDR's (Baron *et al.*, 2016; Bush *et al.*, 2011). Inflow of untreated sewage water, medical waste from hospital environments and industrial effluents into the water bodies makes them one of largest reservoirs of MDR strains. Lack of epidemiological information relating to the dissemination of MDR in the environment, industrial and human sectors requires monitoring (Lupo *et al*., 2012). *E. coli* is a common bacteria found in gastrointestinal tracts of both animals and humans. It is alarming that extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae are spreading quickly in maritime coastal settings because enterobacteria species (mostly *E. coli*) are commensal in the gastrointestinal microbiota of fish (Brahmi *et al.*, 2018; Gerzova *et al.*, 2014). In densely populated nations like India, *E. coli* contamination of seafood occurs as a result of home sewage contamination of coastal waters (Kumar *et al.*, 2004; Prakasan *et al.*, 2018). Gastroenteritis, wound infections, infections of urinary tract, septicaemia and many diseases are caused by this pathogen. Since, ESBL-producing *E. coli* can hydrolyze penicillin, monobactams and broad-spectrum cephalosporins are often resistant to other antimicrobial classes like aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole so, treating infections caused by these bacteria is becoming more difficult (Önnberg *et al.*, 2011). Although animal antibiotic consumption is projected to

increase by 11.5% between 2017 and 2030, human antibiotic usage increased by 65% between 2000 and 2015. If no action is taken, the global usage of antibiotics is predicted to increase by 200% between 2015 and 2030 (Centre for Disease Dynamics, Economics and Policy, 2015). Humans and animals in India live in close contact, increasing the danger of contamination and resistance between them, with 95% of adults in India showing resistance to β-lactams (Walsh *et al.*, 2011). The indiscriminate and increased use of antibiotics in animal husbandry places significant pressure on the gut microbiome acquire resistance due to shorter generation time and high density. Thus, gut acts as a bioreactor for the multiplication of antibiotic resistant bacteria and are continuously discharged into various niches (Dafale *et al*., 2020). Understanding, reviewing and acknowledging the interconnection between human, animal and environment due to the fact that bacteria and genes cross environments and species boundaries. It is challenging to manage such global health problems (Larsson & Flach 2022). Therefore, this study is taken up to evaluate its prevalence.

MATERIAL AND METHODS

Sample Collection, Isolation and Identification of ESBL Producing *E. coli.* Sample collection was carried out between December 2020 to February 2021. The samples were collected from informal fish market in Kalamboli, Navi Mumbai. In this research, fresh fish, fish wastes and its surroundings were considered, as they are the most hazardous foods in terms of food-borne illnesses (Magiorakos *et al.*, 2012). In a span of three months total (n=430) samples were collected.

For sample collection gut swabs of different fish samples like *Tylosurus punctulatus, Asterias amurensis, Scomboromorus commerson, Siluriformes, Selachimorpha, Coilia dussumieri, Anchovy, Engraulidae, Bramidae, Harpadon nehereus, Salmosalar* etc and also swabs from fish associated environment like water samples, table tops, currency, fish monger and swabs, fish waste etc were also collected. Samples were collected using sterile culture collection swabs (HiMedia, Mumbai, India). Swabs collected in sterile conditions were transported to the laboratory in cold chain and processed on the same day for further isolation. Swabs were enriched in 5ml sterile Brain Heart Infusion Broth (BHI) for 24 hours at 37°C. For isolation of ESBL *E coli*, HiCrome agar (HiMedia, India) supplemented with HiCrome ESBL agar supplement (HiMedia, India) was used. The supplement contains ceftazidime, cefotaxime, ceftriazone, aztreonam, fluconazole. Enriched BHI broth was streaked on Hi Crome agar. Colonies showing pink purple color were selected for further studies.

Selected colonies were streaked on Eosin Methylene Blue (EMB) and MacConkey (MAC) agar. *E. coli* colonies shows green metallic shine on EMB agar and pink colonies on MAC agar (Van *et al.*, 2019). Typical bacterial colonies verified morphologically and biochemically by Gram's staining, catalase and oxidase tests, motility at 20–25°C, MR-VP, nitrate reduction, and sugar fermentation (sorbitol, fructose, mannitol and dextrose) (Baron *et al*., 2016; Bush *et al.*, 2011; Olaitan *et al.*, 2015).

Phenotypic Antibiogram of ESBL *E. coli* Isolates. Antibiogram pattern of phenotypically confirmed ESBL *E. coli* strains were subjected to standard disc diffusion method for the Clinical Laboratory Standard Institute Guidelines (CLSI 2018). Fifteen different antibiotic discs (HiMedia, Mumbai, India) representing 09 diverse classes were used (Table 1). The Kirby Bauer method was used to conduct antibiotic susceptibility testing (AST) on Muller Hinton (MH) agar.

Table 1: List of antibiotics used.

Class	Antibiotics
Aminoglycosides	Amikacin (30mcg)
	Gentamycin (30mcg)
Carbapenems	Imipenem (10mcg)
	Meropenem (10mcg)
Cephalosporins (Third Generation)	Cefotaxime (10mcg)
	Ceftazidime (30mcg)
	Cefepime (30mcg)
Penicillin	Amoxycillin (25mcg)
Polypeptide	Colistin (10mcg)
Quinolones / Fluoroquinolones	Ofloxacin (30mcg)
	Nalidixic acid (30mcg)
Tetracycline	Tetracycline (30mcg)
Beta Lactamase inhibitor Antibiotics	Cefotaxime / Clavulanic acid (30/10mcg)
	Ceftazidime / Avibactam) (30/10mcg)
	Amoxyclav (Amoxycillin / Clavulanic acid) (20/10 mcg)

Agar Dilution Method for Determining the Minimum Inhibitory Concentration (MIC) of Cefotaxime. Agar dilution method was carried out according to the CLSI's previously published methods in (M100Performance Standards for Antimicrobial Susceptibility Testing, 2018). Cefotaxime sodium salt from Himedia was used for the study, as it is more bactericidal than the other two antibiotics of 3rd generation cephalosporins (Nath *et al.*, 1995). Protocol for MIC was according to CLSI guidelines. Three stock solutions were prepared (A, B and C) in sterile 500ml bottles and pipetting was done aseptically with different cefotaxime dilutions 0.125 to 1024 µg/ml. A bacterial suspension (overnight growth) equivalent to 0.5 McFarland in 0.9%(w/v) saline was generated and 1:10 diluted 2 µl sample was spotted onto each MH agar plate in duplicate with multichannel pipette, resulting in an approximate final inoculum of 1×10^7 CFU/spot. To prevent variations in inoculum density, plates were inoculated within 30 minutes of inoculum preparation. The lowest antibiotic concentration that prevented bacterial growth was noted as MIC.

Confirmation of ESBL by E-test. Multi – Ezy MIC™ Strips (HiMedia Laboratories Pvt. Ltd.) were used to confirm ESBL production. In Multi-Ezy MIC™ Strips instead of one antibiotic, three antibiotics were employed in a gradient, one side of which contained clavulanic acid whereas the other did not. On one side of the Multi-Ezy MIC™ strips, there was a two-fold gradient of cefepime, cefotaxime and ceftazidime

(MIX) on the other side, the same antibiotics plus clavulanic acid (MIX+). A ratio of inhibition zones for MIX and MIX+ of less than eight was considered positive for the E-test (CLSI 2011). As a negative control, *E. coli ATCC 25922 was used.* Twenty representative cultures were used for E-test.

Multiple Antibiotic Resistance (MAR) Index D. Each isolate's Multiple Antibiotic Resistance (MAR) index was computed using the formula MAR = a/b, where "a" represents the number of antibiotics to which the test isolate shown resistance and "b" represents all antibiotics to which the test isolate's sensitivity has been evaluated (Singh *et al.,* 2020).

$$\text{MAR Index} = \frac{\text{Antibiotics that the isolate was resistant to in terms of number (a)}}{\text{Number of antibiotics used altogether (b)}}$$

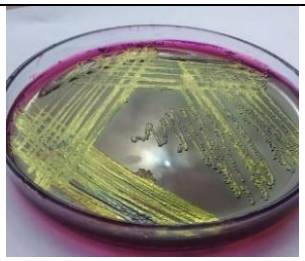
RESULTS AND DISCUSSION

Sample collection, Isolation and Identification of *E. coli.* Within three months total (n=430) samples were collected. Out of 430 samples 60 samples were positive (13.95%) for ESBL *E. coli.* It was evident that more ESBL *E. coli* were isolated from fish waste as compared to the fresh fish samples and associated environment (Table 2).

E. coli were isolated and identified on the basis of cultural morphology on (differential) MAC and (selective medium) EMB agar plates. *E. coli* was characterized by dark colonies with green metallic sheen on EMB medium and pink lactose fermenting colonies on MAC plate. Isolates were gram negative, catalase, indole and methyl red tests positive, nitrate reduction, fermenting sorbitol, fructose, mannitol, dextrose sugars and were motile. Hence, the isolates were confirmed as *E. coli* (Fig. 1a & b). 475 *E. coli* isolated from fresh seafood of which 71.58% were ESBL (Singh *et al.,* 2020). Similar type of work was reported which describes ESBL enterobacteriaceae isolates in sewage water (Said *et al.,* 2020). Another study reported 169 (78.60%) ESBL positive phenotypic *E. coli* (Sanjit *et al.,* 2017).

Table 2: Sample-wise distribution of ESBL+ *E. coli* isolates.

Isolate code	Source
EC1	*Pangasius bocourti*
EC2	Anguilliformes
EC3	*Harpadon nehereus*
EC4	*Xiphias gladius*
EC5	*Taractes miltonis*
EC6	*Platybelon eargealus*
EC7	*Parupeneus indicus*
EC8	*Aristichthyes nobilis*
EC9	*Pampus argnteus*
EC10	*Aphanopus carbo*
EC11 TO EC18	*Platybelone argalus*
EC19	*Coregonus clupeaformis*
EC20 TO 35	*Platybelone argealus*
EC36 and EC 37	*Labeo bata*
EC38 TO 60	Fish waste



(a) *E. coli* on EMB agar showing green metallic sheen

(b) *E. coli* showing pink colonies on MAC agar

Fig. 1.

Phenotypic Antibiogram of ESBL *E. coli* Isolates. Phenotypic antibiogram of all the isolates was performed by Kirby Bauer method. Antibiotic susceptibility testing of isolate EC01 which showed resistance to 14 antibiotics is presented (Fig. 2). Sixty *E. coli* isolates were categorised as sensitive, intermediately resistant and resistant based on the results interpreting following the recommendations of CLSI (Fig. 3) (CLSI, 2018). The antibiotic resistance patterns of 60 ESBL positive isolates are presented (Fig. 4) highest percentage of isolates 98.4 were resistant to amoxicillin whereas all the isolates were susceptible to colistin (Table 4). Of the three cephalosporins only cefotaxime (10mcg) was used for further MIC studies as it has shown only 21.0 % resistance while ceftazidime (30mcg) and cefepime (30mcg) have shown 91.1% and 91.9% respectively. Apart from that cefotaxime is a general basic drug of choice and is frequently used with high efficiency having least side effects.

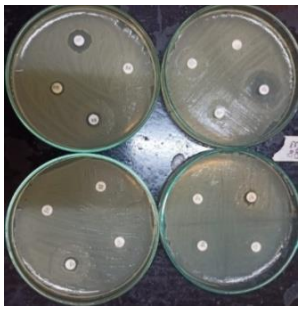


Fig. 2. Antibiotic susceptibility testing of the isolate EC01.

E test and Phenotypic Confirmatory Disc Diffusion test (PCDDT) results showed that 34.71% (68/195) of samples were ESBL producers. It was found that all ESBL producers were imipenem-sensitive (Aruna & Mobashshera 2012). Our reports revealed 100% sensitivity to colistin and more than 90% resistance with three antibiotics. Similar results were observed wherein *E. coli* resistance to important antibiotics cephalosporins and jeopardize the effective prevention and treatment of various bacterial infections (Tamta *et al.*, 2020). 261 (80.30%) ESBL strains isolated from finfish, 79 (52.66%) from shellfish (Singh *et al.*, 2020). Fish processing factory effluents in Mangalore contained ESBL strains that were identified, thereby highlighting the need for treatment of effluents from fish processing plants also highlighted ESBL *E. coli* strains to be a key antimicrobial resistance concern (Day *et al.*, 2019; Divyashree *et al.*, 2019; WHO 2020). In addition, Enterobacteriaceae were reported that produce ESBL and AmpC were found in 21.3% of food samples from Germany that also contained *Klebsiella pneumonia* and *E. coli* (Vu *et al.*, 2018). another study reported that wild fish in Brazil contained *E. coli* that produced ESBL (CTX-M) (Sellera *et al.*, 2018). ESBL In Cambodia, *E. coli* strains were also discovered in chicken, fish, and infected patients (Nadimpalli *et al.*, 2019). Cabello *et al.* (2013) reviewed use of antimicrobials in aquaculture and their associations and their possible risk of transmission antimicrobial resistance in humans through the food chain. All of these publications emphasize the danger of ESBL *E. coli* spreading in areas with substantial seafood consumption. Thereby becoming an eye opener, emphasizing on the need to improve the hygiene of seawater and retail market.

Antimicrobial resistance (AMR) is a growing concern in the field of public health and food safety. While the use of antimicrobial agents in fish farming and aquaculture can help control and prevent bacterial infections, it can also contribute to the emergence and spread of antimicrobial-resistant bacteria. Here are a few examples of studies highlighting the presence of antimicrobial-resistant bacteria in fish.

Presence of antibiotic resistance genes (ARGs) in aquaculture environments and their potential transfer between bacteria. It found that fish farms served as reservoirs of ARGs, and these genes could be transferred between bacteria, including pathogenic strains (Zou *et al.*, 2017). Previous europian studies reported the CTX-M-15- the dissemination of this resistant strain, highlighting the potential transmission of resistant bacteria through the global seafood market (Rodriguez *et al.*, 2009).

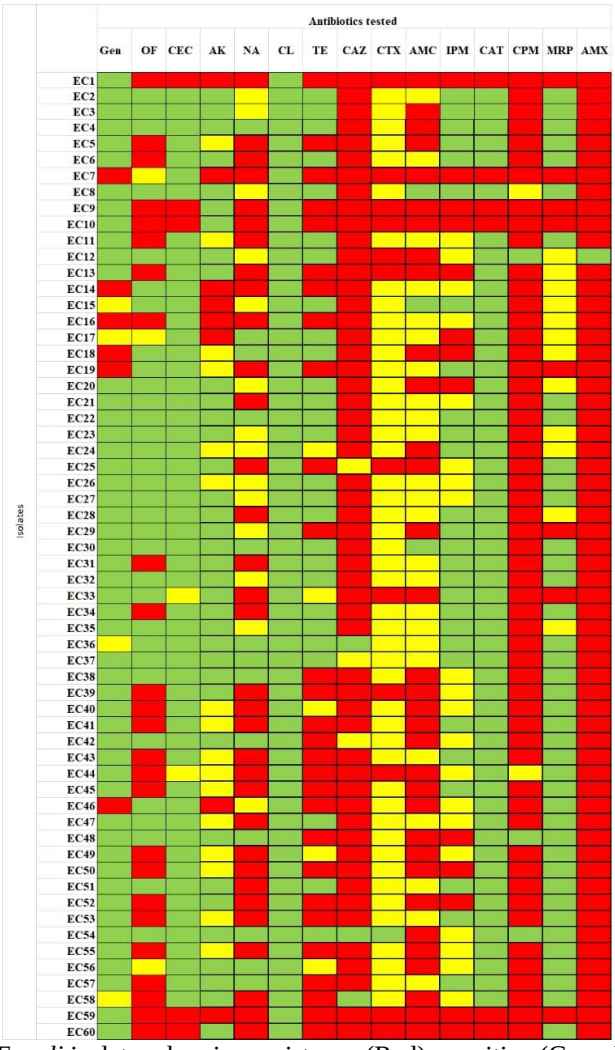


Fig. 3. Characteristics of *E. coli* isolates showing resistance (Red), sensitive (Green) and Intermediate (Yellow).

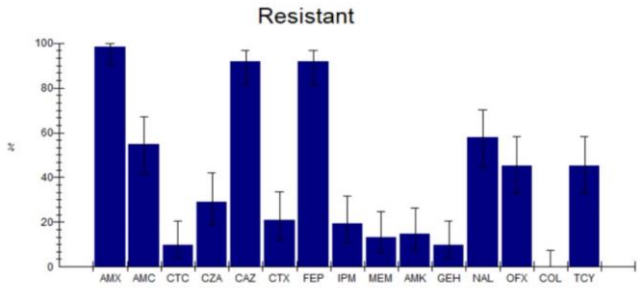


Fig. 4. Resistance pattern of 60 isolates against 15 antibiotics.

AMX- Amoxicillin, AMC- Ampicillin, CTC-Cefotaxime/Clavulanic acid, CZA-Ceftazidime/Avibactam, CAZ-Ceftazidime, CTX-Cefotaxime, FEP- Cefepime, IPM- Imipenem, MEM-Meropenem, AMK- Amikacin, GEH- Gentamycin, NAL- Nalidixic acid, OFX- Ofloxacin, COL- Colistin, TCY- Tetracyclin.

Minimum Inhibitory Concentration Studies (MIC). Results of MIC were interpreted and MIC values of different isolates were studied (CLSI 2018). Of the 60 isolates only isolate (EC 48) showed sensitivity with MIC of 8µg/mlto cefotaxime (Table 5). It is clear that 87% of the isolates had MICs greater than 1024 µg/ml to cefotaxime, while EC-03, 14, 28 and 30 showed MIC value of 512µg/ml, while EC-11 showed 256 µg/ml, EC-44 and 54 showed 128µg/ml and 64µg/ml respectively. Majority of the bacterial isolates from cattle beef were cefotaxime resistant (concentrations > 64 µg/ml) and had high multi-drug resistance (Mir *et al.*, 2016). In one study the MIC was reported more than 256mcg/ml for general antibiotics.

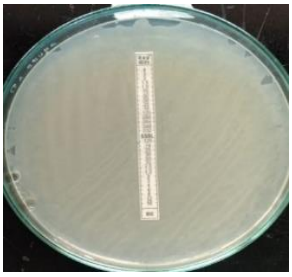


Fig. 5. E-test of EC01 by Multi-Ezy MIC™ Strips.

Confirmation of ESBL Production by E-test. Of the 60 isolates, confirmation of ESBL producers by E-test was done for only 20 isolates. All these 20 isolates tested by E-test showed resistance against Multi-Ezy MIC™ Strips due to the production of ESBL enzyme. E-test of only EC01 is depicted (Fig. 5). Confirmatory test for ESBL producer were reported using MIC™ Strip which showed 34.87% to be ESBL producers whereas our studies revealed 100% resistance to MIC™ (Aruna & Mobashshera 2012).

Determination of MAR Index. All 60 isolates had a MAR score of > 0.18, indicating contamination from high-risk sources including locations where antibiotics are often used. These isolates were all resistant to two or more antibiotics. The MAR index ranged from 0.2 to 0.87 in our results (Table 6), indicating sources with the greatest contamination levels are high risk. Thus, this category encompassed all 60 isolates found in fresh fish, fish waste, and nearby locations.

P. penneri had the highest MAR index (0.87) whereas *C. freundii* and *E. cloaca* had the lowest (0.22%), illustrating the significant pressure for antibiotic selection in the gold fish farm (Prenna *et al.*, 2020). The health risk associated to the spread of resistance is assessed using the MAR index. Aquaculture suffers from severe antibiotic use when the MAR index is higher than 0.2% (Abdalla *et al.*, 2021). The high prevalence of ESBL E. coli strains in coastal water is highlighted by the fact that all of the isolates had MAR indices >0.20 ranging from 0.20 to 0.87 and a mean value of 0.53, and our findings are consistent with this finding, showing that all isolates had MAR indices >0.20 ranging from 0.21 to 0.85 and a mean value of 0.45. A MAR score of greater than 0.2 implies that antibiotics are heavily used in aquaculture or are ingested heavily from other sources (Krumperman, 1983).

Table 3: Antibiotic susceptibility patters of ESBL positive *E. coli* (n=60) isolated in this work.

Tested Antibiotics	Resistant (%)	Intermediate Resistant (%)	Susceptible (%)
L5Amikacin (amk) 30 mcg	14.5	24.2	61.3
Gentamycin (GEN)30 mcg	9.7	9.7	80.6
Imipenem (IPM)10 mcg	19.4	35.5	45.2
Meropenem (MEM)10 mcg	13.1	19.7	67.2
Cefotaxime (CTX)10 mcg	21	77.4	1.6
Ceftazidime (CAZ)30 mcg	91.1	4.8	3.2
Cefepime (FEP)30 mcg	91.9	3.2	4.8
Amoxycillin (AMX)25 mcg	**98.4**	**0**	**16**
Colistin (COL)10 mcg	**0**	**0**	**100**
Ofloxacin (OFX)30 mcg	45.2	6.5	48.4
Nalidixic acid (NAL)30 mcg	58.1	24.2	17.7
Tetracycline (TCY)30 mcg	45.2	9.7	45.2
Cefotaxime / Clavulanic acid (CTC)30/10 mcg	9.7	4.8	85.5
Ceftazidime / Avibactam (CZA)30/10 mcg	29	0	71
Amoxyclav (Amoxycillin / Clavulanic acid (AMC)20/10 mcg	9.7	4.8	85.5

Table 4: MIC values and its interpretation of all 60 isolates for Cefotaxime.

Isolate code	MIC value (µg/ml)	Interpretation
EC1,2,4,5,6,7,8,9,10,12,13,15,16,17,18,19,20,21,22,23,24,25,26, 27,29,31,32,33,34,35,36,37,38,39,40,41, 42,43,45,46,47,49,50,51,52,53,55,56,57,58,59,60	1024	Resistant
EC3,14,28,30	512	Resistant
EC11	256	Resistant
EC44	128	Resistant
EC48	8	Sensitive
EC54	64	Resistant

Table 5: *E. coli* Multiple Antibiotic Resistance (MAR) Indices.

MAR Index	Isolates code	No. (%) of isolates
0.2	EC54	1(1.67%)
0.26	EC30	1(1.67%)
0.33	EC4, EC8, EC36, EC37, EC22	5(8.33%)
0.40	EC2, EC3, EC12, EC32, EC48, EC 51	6 (10.0%)
0.46	EC21, EC20, EC27, EC28, EC31	5(8.33%)
0.47	EC6, EC23, EC34,EC35,EC38, EC42, EC57	7(11.67%)
0.53	EC15, EC25, EC26, EC47, EC56	5(8.33%)
0.60	EC5, EC11, EC18, EC24, EC33, EC39, EC43, EC45, EC53, EC 58, EC52	11(18.33%)
0.66	EC55	1(1.67%)
0.67	EC13, EC14, EC17, EC19, EC40, EC41, EC46, EC49, EC50	9(15.0%)
0.73	EC44, EC60	2(3.^{33}%)
0.80	EC9, EC10, EC16	3(5.0%)
0.87	EC1, EC7, EC59	3 (5.0%)

CONCLUSIONS

Studies on antimicrobial resistance in *E. coli* from food animals thereby contaminating humans has drawn special attention towards migration of resistance gene from animal to human through close association with animal reservoirs is limited and no such deep cohort studies in India are prevailing. Such studies are of urgent need due to the environment and public health concern. Pathogen resistance to new generation cephalosporins could present significant difficulties. To stop the spread of antibiotic resistance in coastal waters in such a scenario, efficient management measures must be employed. These studies therefore highlight the requirement for a thorough analysis of the application of antibiotics and growth promoters in the food and animal production chains.

FUTURE SCOPE

The analysis of antibiotic-resistant bacteria in fish products is an important area of research and surveillance, as it helps identify potential risks to human health and informs strategies for antimicrobial stewardship. Antibiotic resistance occurs when bacteria develop the ability to withstand the effects of antibiotics, rendering them ineffective in treating bacterial infections. The findings of present investigation can inform regulatory agencies, policymakers, and the food industry to implement appropriate measures for the control and prevention of antibiotic-resistant bacteria in fish products. It may involve improving aquaculture practices, implementing stricter regulations on antibiotic use, promoting responsible antimicrobial stewardship, and ensuring proper food safety protocols throughout the production and supply chain.

Authors contribution. Concept and design: NR, RV, DD; Data acquisition: NR, TN, JS; Data analysis / interpretation: NR, JS, NT, DD; Drafting manuscript: NR, JS, DD; Critical revision of manuscript: NR, RV, TN, JS, DD; Supervision & final approval: RV.

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Conflict of Interest. None.

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