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SCREENING FOR EXTENDED-SPECTRUM BETA-LACTAMASES PRODUCING *ESCHERICHIA COLI* ISOLATES IN FISH SAMPLE AND PROFILING FOR ANTIBIOTICS SUSCEPTIBILITY

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ABSTRACT

At present, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and other species of Enterobacteriaceae have emerged as a matter of utmost concern. Indiscriminate utilization of antimicrobials in aquaculture generates selective pressure creating reservoirs of drug-resistance genes in fish pathogens and other bacteria, which may disseminate by horizontal gene transfer and reach human pathogens. The present study aims to detect extended-spectrum beta-lactamases (ESBL) production by enterobacteria isolated from locally harbored and imported fresh and dry fishes.
Out of 235 fish samples investigated, the observed incidence of 9.78% (n=23) *E. coli* isolates. PCR detection of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* using primers specific for *Bla-TEM, Bla-SHV, Bla-CTX-M.* As per molecular detection by Multiplex PCR, 11 isolates exhibited Bla-$TEM^{+}CTX\text{-}M^{+}SHV$ genes, and two isolates exhibited the presence of *TEM* genes only. Antimicrobial susceptibility patterns against 12 antibiotics were studied, where it was observed that resistance to the selected drug ranges from 4.34 % to 73.9 % for 12 different antibiotics.

Keywords: ESBL, *E. coli*, fish, antimicrobial susceptibility, Multidrug resistance

INTRODUCTION

The current era, where people are developing more obsessions for healthy living, has gained safety standards and microbiological attributes of various food items huge attention, which tends to expand at a very gradual pace **(Patterson 2012)**. Fish serve as a convenient and safe source of essential nutrients-proteins, vitamins, and minerals, making itself an interim part of the staple diet, supplementing the maximal part of all nutrient needs **(Abdullahi *et al.*, 2001)**.
Numerous studies have been conducted in various parts of India by researchers to check the bacteriological quality of freshly landed and retail market fish and other seafood **(Lakshmanan *et al.*, 1988)**. Reports have emerged about the deplorable quality of fishes available in domestic markets that are mostly contaminated by pathogenic microorganisms compared to export quality **(Nambiar *et al.*, 1990)**. The contamination of water bodies and estuaries by fecal contamination due to poor sanitary conditions and activities by the adjoining populations and deposition of untreated sewages is emerging as one of the alarming issues concerning fishes' quality calls for strict vigilance and maintenance **(Patterson, 2012)**. The low maintenance and unhygienic conditions of the landing and storage centers and market places exacerbate poor hygiene and consumer safety of fishes **(Kumar *et al.*, 2005)**.
Escherichia coli is widely distributed in the environment as a dominant and innoxious gut flora of humans and animals. In contrast, certain strains of E. coli acquire pathogenicity via procuring virulence factors or through the spread of resistant genes causing intestinal and extraintestinal infections **(Bielaszewska *et al.*, 2011)**, diarrheal infections, gastroenteritis **(Jawetz *et al.*, 1984; Levine, 1987)**, posing a threat of transferring antibiotic resistivity to other clinically essential strains **(Kumar, 2017)**. *E. coli* strains pathogenic towards human beings have evolved as a significant cause of a wide range of diseases than any other pathogenic organism **(Nataro and Kaper, 1998; Paton and Paton, 1998)**.
Due to poor hygiene in the rapidly blooming fish farming and harvesting sector **(Sapkota *et al.*, 2008)**, extensive use of antimicrobial agents potentially contributes to increased resistant strains **(Benbrook, 2002)**, threatening humankind, impacting fish farming and their environment. Also, irrational use, coupled with the transmissibility of resistance, marks the emergence of new ESBL producing *E. coli* **(Cantos *et al.*, 2016)**.
Emergence and desiccation of ESBL producing Enterobacteriaceae since the last decade **(Bradford, 2001; Paterson & Bonomo, 2005; Bush, 2008; Ozcakar *et al.*, 2011)** along with the emergence of Multidrug resistance (MDR) among such strains are rising as an alarming rate **(Cheng *et al.*, 2014)**. Prevalence of ESBL producing *E. coli* strains has been witnessed in most livestock-oriented foods and food products - meat, chicken, fish, raw milk, and milk products **(CLSi, 2006; Egea *et al.*, 2012; FSSAI, 2012; Elhadi and Alsamman, 2015)**. Reports about fish being the reservoir of ESBL producing *E. coli* strains have also been found in China **(Jiang *et al.*, 2012)**. Explicitly, extended-spectrum Beta-Lactamases are enzyme coded by plasmid confined within strains with a four-membered ring, making them capable of inactivating a broad range of β-lactam antibiotics broad-spectrum monobactams and cephalosporin **(Bush *et al.*, 1995; Paterson & Bonomo, 2005)**. Numerous research has revealed the emergence of ESBLs as mutations in Sulphydryl variable (bla-SHV), Temoneira (bla-TEM) genes followed by substitution mutation in the active site the protein level producing TEM and SHV type of *E. coli.* Certain strains also synthesize an additional enzyme CTX-M (Cefotaximase-Munchen), producing bla-CTX-M **(Manoharan *et al.*, 2011)**, which is more active against 3G Chephelosporin antibiotics, i.e., cefotaxime and ceftriaxone, than ceftazidime. However, point mutations could increase their action against ceftazidime too. CTX-M has been extensively described in different livestock animals and food products recognized as a reservoir of ESBL producing *E. coli* strains **(Carattoli, 2008; Geser *et al.*, 2011; Egea *et al.*, 2012)**. Numerous reports have suggested that antibiotic resistance genes (bla-CTX-M, bla-SHV, bla-TEM) mostly divulge via food chains or by through interaction with humans and animals **(Oppegaard *et al.*, 2001; Winokur *et al.*, 2001; Mesa *et al.*, 2006; Egea *et al.*, 2012)** making the microbiological well-being of edibles-fish and fish produces a point of concern all over the world **(FAO, 2010)**.
The threat due to enteric pathogens in fishes becomes more confounding when such bacteria are multidrug-resistant as there is no data available about antibiotic sensitivity pattern related to ESBL-producing bacteria in aquaculture products in Silchar, Assam, India. The current study was intended to detect the occurrence of *E. coli*, observing the antimicrobial susceptibility, presence of Multidrug resistance, and finally genotypic characterization of the isolates to check the prevalence of ESBL producing *E. coli* in the fishes procured from local retail and wholesale outlets in Silchar, Assam, India.

MATERIAL AND METHODS

Sample collection

In the present study, 235 samples of fishes were randomly taken from Silchar's various retail and wholesale markets during May-July, 2018. The samples were collected in sterile sample bags using sterile hand gloves and were immediately transported to the laboratory in a refrigerated container and processed for bacterial isolation within 24 hours.

Bacterial isolation and analysis

Aseptically procured samples were gently washed with distilled H_2O and 5g of sample added to 45ml of Alkaline Peptone Water and incubated overnight at 37°C followed by streaking onto Eosin Methylene Blue Agar (Hi media) plates; nucleated colonies showing green metallic sheen were subcultured on MacConkey Agar For selective and differential isolation of *E. coli.* Suspected colonies were confirmed by the IMViC test **(Montenegro *et al.*, 1990)**. Biochemically confirmed isolates were stored in Luria broth: glycerol (1:1) stock at -20°C for further analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was accomplished in accord with CLSI guidelines. Antimicrobial agents were tested against the isolates following Kirby-Bauer's disc diffusion technique on Mueller-Hinton agar plates, and plates were incubated overnight at 37°C. Distinct regions of inhibition were measured in mm and deciphered into Susceptible (S), Intermediate (I), and Resistant (R) groupings as per NCCLS guidelines. Antibiotics and their potencies **used are listed in table 1**.

Table 1 antibiotics and their potencies used in the study.

Antibiotics	Potencies (mcg)
Ampicillin	10
Amikacin	10
Cefotaxime	10
Ciprofloxacin	10
Gentamicin	50
ImipenemEDTA	10/10
Streptomycin	25
Ofloxacin	2
Ceftriaxone	30
Cefepime	30
Piperacillin/Tazobactam	100/10
Meropenem	10
Ampicillin/Sulbactam	10/10
Levofloxacin	5

Detection of **ESBL production**

Isolates exhibiting a diameter of less than 27 mm for Cefotaxime and less than 25 mm for Ceftriaxone in antibiotic sensitivity testing were subjected to ESBL production test as per Modified Double Disc Synergy test, by placing a disc of Amoxicillin-Clavulanate (20/10mcg) at the center along with three Cephalosporins viz, Cefotaxime and Ceftriaxone (3GC), Cefepime (4GC), 15mm and 20mm away respectively from center to center of Amoxicillin-Clavulanate disc on a lawn culture of test organism on MHA plates (as per CLSI guidelines) and observed for any contortions or growth of zone of inhibition to the Amoxicillin-Clavulanate disc.

DNA extraction

Genomic DNA was isolated from 5ml of LB broth culture as per the manufacturer's protocol (Genomic DNA Miniprep Kit, mdi Membrane Technologies (India), and extracted DNA was stored at -20°C. 1-2 µl of genomic DNA (200ng/µl) was used as a template for PCR, according to **Ozpinar *et al.* (2013)**.

Polymerase chain reaction

For amplification of β-Lactamase genes, oligonucleotide primers of *Bla-TEM, Bla-SHV, and Bla-CTX-M* were used **(Ozpinar *et al.*, 2013)**. PCR was performed in 25µl reaction mixture containing 2.5µl of Taq buffer (10X), 0.5µl dNTPs (10µm), 1µl of each primer (10 picomoles), 2µl of template DNA (200ng/µl), 1 unit of Taq DNA Polymerase (NEB), and sterile MQ H_2O were used to make the final volume of 25µl. PCR was performed for 32 cycles at 95°C for 5 minutes (Initial denaturation), 95°C for 45 seconds (Denaturation), 52.4°C for 45 seconds (Annealing), 72°C for 1 minute (Extension), and a final extension at 72°C for 7 minutes. Amplified PCR products were resolved in 1% Agarose gel.

RESULTS AND DISCUSSION

Screening of a total of 235 fish samples, including freshly harbored, dried and inland-imported fish, exhibited an overall incidence of 9.78% (n=23) biochemically confirmed *E. coli* isolates (Table 2).

A similar study comprising raw fish samples and ready to eat fish products from Ludhiana, India, revealed an overall incidence of 47 and 7 *E. coli* isolates from raw fishes and ready to eat fish products, respectively **(Gupta *et al.*, 2012)**. A study carried out by **Kumar et al. (2005)** revealed that 38.8% of finfish samples from the fresh fish market and 25% from landing centers were confirmed as *E. coli* isolates. A study performed by **Thampuran et al. (2005)** in Veraval district of Gujarat on seafood from the retail fishery outlets for the prevalence of ESBL fabricating *E. coli* revealed the occurrence of *Escherichia coli* isolates in 28 samples out of 238, i.e., approx 12%, firmly supporting the findings of the current study. From a study carried out by **Dutta et al. (2016)** to find out the prevalence of *E. coli* in shrimps and fishes sold in retail markets at Kolkata, India kept forward the occurrence of the bacteria in 80.70%, i.e., 138 samples out of 171 studied samples using standard microbiological and biochemical tests. **Ryu et al. (2012)** studied during the period 2005 to 2008 based on 2663 marine products sold in Seoul's retail and wholesale markets, Korea revealed 179 (6.7%) samples as the bearer of the concerned organism supporting the findings of the current study. **Jeyasanta et al. (2012)** studied a total of 168 samples collected from the main landing centers of intertidal and fish markets of Tuticorin coast, out of 128 samples appearing to be positive for the presence of fecal coliform, 91, i.e., 71.8% of the value appeared as positive as *E. coli*.

Table 2 Incidence of *E . coli* in fresh and dry fish samples from various Silchar markets

Area of the collected sample	No of the samples collected	Incidence of *E. coli* in samples	% of *E. coli* in the samples
Ithkhola Market	55	3	5.45
National Highway Market	50	2	4
Madhuraghat Market	31	0	0
Door-to-door vendors	32	3	9.37
NH Chourangi Market	12	5	41.66
Fhatakbazar Market	21	3	14.28
Annapurna market+Sanjay Market	18	4	22.22
College Road Market + SMC Market	16	3	18.75
Total	**235**	**23**	**9.78**

Antibiotic Susceptibility Pattern of *E. coli* isolates

In the current study, antimicrobial susceptibility patterns contrary to 12 antibiotics were studied for 23 *E. coli* isolates where it was observed that resistance to the selected drug tested ranges from 4.34% to 73.9% for 12 different antibiotics. In-vitro antimicrobial susceptibility test results have been noted in Table 3. Furthermore, out of 23 positive isolates, 11 showed resistance to more than two antibiotics used in the susceptibility assay and can be considered Multidrug-Resistant (MDR).

Table 3 In-vitro antimicrobial susceptibility test result

Antibiotics	No. of resistant isolates	Percentage of resistant isolates	No. of susceptible isolates	Percentage of susceptible isolates	No. of intermediately resistant isolates	Percentage of intermediately resistant isolates
Ampicillin	16	69.6	-	-	07	30.5
Amikacin	01	4.3	22	95.7	-	-
Cefotaxime	12	52.2	08	34.8	03	13
Ciprofloxacin	10	43.5	11	47.8	02	8.7
Gentamicin	5	21.8	15	65.2	03	13
Imipenem EDTA	01	4.3	22	95.7	-	-
Streptomycin	-	-	23	100	-	-
Ofloxacin	15	65.2	06	26.1	02	8.7
Ceftriaxone	11	47.8	06	26.1	06	26
Cefepime	15	65.2	06	26.1	02	8.7
Piperacillin/Tazobactam	13	56.6	09	39.1	01	4.3
Meropenem	17	73.9	05	21.8	01	4.3
Ampicillin/Sulbactam	03	13	20	87	-	-
Levofloxacin	05	21.8	15	65.2	03	13

A study based on finding *E. coli* in the meat of shrimp, fish, chicken, and mutton at Vishakhapatnam, Andhra Pradesh, reported massive E. coli presence in the samples and 90% resistance towards ampicillin **(Chakravarty *et al.*, 2015)**. In the study of Veraval, Gujarat, the 28 samples found to be positive as *E. coli* when subjected to antibiotic sensitivity checking showed the highest rate of resistance towards ampicillin (39.29%), 11 samples appeared as MDR. In contrast, 4 (14.29%) were confirmed as ESBL producers, supporting the findings of the current study of 16 out of 23, i.e., 69.56% of *E. coli* isolates being resistant to ampicillin, 12 being MDR, and 14 being ESBL producers **(Sivaraman *et al.*, 2017)**. **Ryu HS *et al.* (2012)** study showed the highest resistance towards tetracycline (30.7%) and lowerest towards ampicillin (6.7%), contradicting the findings of the current study. In a study conducted by **Kumar *et al.* (2005)**, wherein 116 samples of *E. coli* were treated with 14 different antibiotics, seven samples were observed to be resistant to more than five antibiotics, whereas 1 sample resistant to 8 antibiotics establishing shreds of evidence for the existence of Multidrug-resistant *E. coli* in seafood of India. A study by **Jeyasanta *et al.* (2012)** involving 168 *E. coli* samples collected from the Tuticorin coast's fish markets was tested for drug susceptibility against 15 antibiotics; 11 isolates exhibited multiple resistance to 4 while one isolate revealed resistance against five antibiotics.

Polymerase chain reaction

Multiplex PCR was performed to examine the presence of β-Lactamase genes in 23 E. coli isolates; **Bla-*TEM+CTX-M+SHV*** genes were present in 11 isolates; two isolates were harboring only ***TEM*** genes. Results are listed in Table 4 and Figure 1.

Table 4 Frequency of occurrence of ESBL genes in *E. coli* isolates

Market area	No of *E. coli* isolates	Bla-TEM	% of Bla-TEM	Bla-SHV	% of Bla-SHV	Bla-CTX-M	% of Bla-CTX-M	Bla- TEM+ CTX-M+ SHV	% of Bla- TEM+ CTX-M+ SHV
Ithkhola Market	3	2	66.6	1	33.3	1	33.3	1	33.3
National Highway Market	2	1	50	1	50	1	50	1	50
Madhuraghat Market	0	0	-	0	-	0	-	0	-
Door-to-door vendors	3	1	33.3	1	33.3	1	33.3	1	33.3
NH Chourangi Market	5	3	60	2	40	2	40	2	40
Fhatakbazar Market	3	1	33.3	1	33.3	1	33.3	1	33.3
Annapurna market+Sanjay Market	4	3	75	3	75	3	75	3	75
College Road Market + SMC Market	3	2	66.6	2	66.6	2	66.6	2	66.6
Total	**23**	**13**	**56.5**	**11**	**47.8**	**11**	**47.8**	**11**	**47.8**

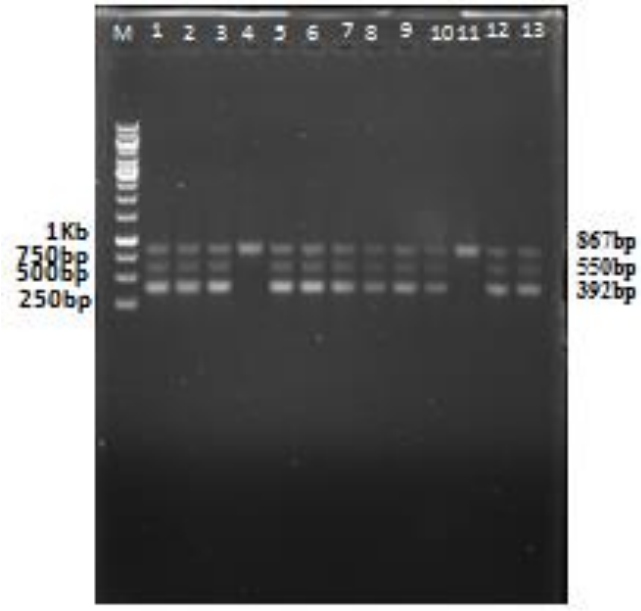

Figure 1 Presence of TEM, SHV, CTX-M gene in *E. coli* by multiplex PCR. Lane 1:1kb ladder, lane 2-13 isolates.

CONCLUSION

The present study outcomes indicate a moderate *E. coli* in fish samples procured from Silchar's different markets. Further analysis indicated a wide variation in their antibiotic sensitivity, along with exhibiting the incidence of a few multidrug-resistant strains and a wide occurrence of ESBL genes (bla-TEM, bla-SHV, and bla-CTX-M). This indicates the necessity to understand the need to suppress human pathogens, the judicious use of antibiotics in aquaculture farms and import lots, substantial level oligotrophication of the water bodies, and finally, the need to bring these regulatory and educational efforts into action to maintain safety and quality of both fishes and the consumers.

Conflict of interest: The authors declare that no conflict of interest exists.

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