Prevalence and molecular characterisation of extended-spectrum \(\beta\)-lactamases genes in *Escherichia coli* and *Salmonella* spp. isolated from fish samples in Chhattisgarh, India

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**ABSTRACT**

In this study, fish samples were examined for the presence of extended-spectrum \(\beta\)-lactamases (ESBL) producing *Escherichia coli* and *Salmonella* spp. Out of 200 samples collected from retail markets of Durg, Raipur, Rajnandgaon and Balod districts of Chhattisgarh State, 76 (38) and 46 (23%) samples were found contaminated with *E. coli* and *Salmonella* isolates, respectively. Multiple antibiotic resistant (MAR) index varied between 0.00 to 0.88 and 0.11 to 0.88 for *E. coli* and *Salmonella* isolates, respectively. The isolates were screened phenotypically for ESBL production and multiplex PCR targeting \(bla_{TEM}\), \(bla_{SHV}\) and \(bla_{CTX-M}\) genes, was carried out followed by sequencing. Prevalence of \(bla_{TEM}\), \(bla_{SHV}\) and \(bla_{CTX-M}\) genes in *E. coli* and *Salmonella* isolates were found to be 13.2, 1.3, 0 and 6.5, 0, 4.4%, respectively. Higher percentage of resistant genes was detected in plasmid DNA in comparison to genomic DNA. Sequencing of PCR amplicons of \(bla_{TEM}\) and \(bla_{CTX-M}\) genes revealed the presence of \(bla_{TEM-116}\) and \(bla_{CTX-M-15}\) variants, respectively. The study indicates the presence of multidrug resistant ESBL producing *E. coli* and *Salmonella* in fish samples, which may pose threat to the fish consumers.

**Keywords:** \(bla_{TEM}\), \(bla_{SHV}\), \(bla_{CTX-M}\), *E. coli*, Fish, MAR index, *Salmonella*

**Introduction**

Food borne diseases are a major human health burden leading to high morbidity and mortality all over the globe. The problem is more severe in developing countries and it is estimated that 70% of diarrheal diseases in such countries are associated with the consumption of contaminated food due to poor hygienic handling practices. Contamination of fish with potential food poisoning pathogens such as *Escherichia coli* and *Salmonella* spp. can result in adverse effects on human health (Abebe et al., 2014). *E. coli* and *Salmonella* spp. are part of normal intestinal flora of human and animals and ubiquitous in almost all types of soil, water and vegetation. These bacteria cause a variety of human diseases (Murray et al., 1998) and identified as important food-borne pathogens with significant morbidity and mortality (Akkina et al., 1999). The occurrence of extended spectrum \(\beta\)-lactamase (ESBL) producing *E. coli* and *Salmonella* spp. has become a global problem over the past decade (Ozcakar et al., 2011). Among non-typhoidal *Salmonella*, most ESBL-producing strains are amazingly uncommon around the world. The ESBLs are plasmid-encoded enzymes that counteract a substantial number of \(\beta\)-lactam antibiotics, such as extended-spectrum, broad-spectrum cephalosporins and monobactams. Among various ESBLs found in Gram-negative microbes, TEM, SHV and CTX-M types are of major clinical concern and encoded by \(bla_{TEM}\), \(bla_{SHV}\) and \(bla_{CTX-M}\) genes, respectively (Ehlers et al., 2009). The major part of ESBLs are derived from the amino acid substitutions of their parent proteins TEM and SHV \(\beta\)-lactamases, however few different kinds of acquired ESBLs are also found in *Enterobacteriaceae* (Reglier-Poupet et al., 2008).

The emergence of ESBL producing *E. coli* and *Salmonella* spp. in aquaculture is a growing problem worldwide; however literature/reports on the association of these ESBL producing bacteria in aquaculture in India particularly in the state of Chhattisgarh are not available. Therefore, this study was conducted to find out the status of ESBL-producing *E. coli* and *Salmonella* spp. in fish samples of retail markets of Chhattisgarh, India.

**Materials and methods**

**Sample collection**

A total of 200 fish samples were collected randomly from retail fish shops of Durg, Raipur, Rajnandgaon...
and Balod districts of Chhattisgarh, India during August 2017 and July 2018. All the fish samples were aseptically collected following the protocol of International Commission on Microbiological Specifications for Foods (ICMSF, 1978).

**Isolation and biochemical characterisation of E. coli and Salmonella**

Isolation of *E. coli* was carried out as per the method outlined by Meiyarasi et al. (2017) with slight modifications. Briefly, 25 g of fish sample was taken, blended and discharged in 225 ml of MacConkey broth (HiMedia, India) and incubated at 37°C for 24 h. Thereafter, bacterial culture was streaked over MacConkey agar (HiMedia, India). The pink coloured colonies were then picked up and inoculated on EMB agar (HiMedia, India). On EMB agar colonies showing metallic sheen were considered as *E. coli*.

Similarly, isolation of *Salmonella* spp. was carried out as per standard ISO 6579: 2017 guidelines (ISO, 2017) with slight modifications. Briefly, 25 g of fish sample was taken and inoculated in sterilised tetraphionate broth (HiMedia, India) for enrichment. Thereafter enriched culture of *Salmonella* was purified using Brilliant Green Agar (BGA) and Bismuth Sulphite Agar (BSA) (HiMedia, India). On BGA, moderately large, moist, smooth and pink colonies with red background, whereas on BSA black colony surrounded by brownish-black zone with metallic sheen were considered as *Salmonella* species (Nagappa et al., 2007). *E. coli* and *Salmonella* isolates were characterised by biochemical tests viz., indole, methyl red (MR), Voges-Proskauer (VP), Triple sugar iron (TSI), urease and citrate utilisation. All *E. coli* and *Salmonella* isolates were further confirmed by serotyping from National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh.

**Antimicrobial susceptibility test and multiple antibiotic resistance (MAR) index**

*E. coli* and *Salmonella* isolates were tested for their antimicrobial drug susceptibility pattern on Mueller-Hinton agar (MHA) (HiMedia, India) by the disc diffusion method (CLSI, 2012). The antibiotics used were oxytetracycline (30 μg), cephalexin (30 μg), ciprofloxacin (5 μg), gentamicin (30 μg), cefotaxime (10 μg), ampicillin (10 μg), cefazidime (30 μg), aztreonam (30 μg) and cefixime (5 μg) (HiMedia, India). Complete inhibition zone diameter was measured and compared with the interpretation chart (HiMedia, India). The complete inhibition zones were graded as resistant, intermediate and sensitive and MAR index was calculated (Krumperman, 1983).

**Phenotypic detection of ESBL producers**

*E. coli* and *Salmonella* isolates with a zone of inhibition of ≤17 mm for aztreonam and ceftazidime and ≤22 mm for cefotaxime in disc diffusion susceptibility testing were selected for detection of ESBLs production (CLSI, 2012). For this purpose, cefotaxime (10 μg) and cefotaxime+clavulanic acid (30+10 μg) discs (HiMedia, India) were used. The *E. coli* and *Salmonella* isolates showing more than 5 mm dia zone of inhibition were considered as presumptive ESBL producers (Krumperman, 1983).

**Extraction of plasmid and genomic DNA**

*E. coli* and *Salmonella* isolates were subcultured in Luria-Bertani (LB) broth (HiMedia, India) and incubated for 12-16 h prior to extraction. Plasmid was extracted from each isolate using commercially available plasmid extraction kit (HiMedia, India). Genomic DNA was prepared by boiling and snap chill method. Extracted plasmid and genomic DNA were subjected to agarose (0.8%) gel electrophoresis and visualised under UV transilluminator after staining with ethidium bromide.

**PCR**

All the presumptive ESBL producing *E. coli* and *Salmonella* isolates were screened for the presence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes by multiplex-polymerase chain reaction (m-PCR) following the protocol given by Monstein et al. (2007). The oligonucleotide primers specific for the *bla*<sub>SHV</sub> (Paterson et al., 2005), *bla*<sub>TEM</sub> (Apaka et al., 2010) and *bla*<sub>CTX-M</sub> (Boyd et al., 2004) genes were used in the m-PCR assay (Table 1). All the primers used in the present study were procured from the Imperial Life Sciences Pvt. Limited, Gurgaon, Haryana, India. PCR reactions were performed by using 25 μl PCR buffer (Tris with 15 mM MgCl<sub>2</sub>), deoxyribonucleotide triphosphate (25 μM), 1U Taq polymerase, 3 μl of template DNA (genomic/plasmid DNA) and gene specific primers (10 pmol each). The m-PCR amplification was performed with initial denaturation at 95°C for 10 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min followed by a final extension step at 72°C for 10 min. Amplified PCR product was electrophoresed using 1.5% agarose gel, stained with ethidium bromide and examined and recorded using a Gel Documentation System (Gel Doc™XR, Biorad, USA).

**DNA sequencing analysis and nucleotide sequence accession numbers**

The PCR products of randomly selected isolates were further confirmed by Bi-directional nucleotide sequencing of the plasmid DNA (Eurofins Genomics India Pvt Ltd., Bengaluru). PCR products of two *bla*<sub>TEM</sub> genes
of E. coli and two bla\textsubscript{CTX-M} genes of Salmonella isolates were sequenced. Obtained sequences were subjected to blast analysis and similar sequences of different resistant bacterial strains were retrieved from National Centre of Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The partial coding sequence (CDS) of \textit{bla\textsubscript{TEM}} gene of strain \textit{E. coli} R9 and \textit{E. coli} R12 were submitted to GenBank under the accession numbers MN052808 and MN052809, respectively. Similarly, the partial CDS of \textit{bla\textsubscript{CTX-M}} gene of strain \textit{Salmonella} R26 and \textit{Salmonella} R17 were submitted to GenBank under the accession numbers MN052811 and MN052812, respectively. Sequences of nucleotide and deduced protein were compared with other resistant bacterial strains and the degree of similarity was established using the DNASTAR MegAlign software.

**Results**

\textit{Isolation, identification and antimicrobial susceptibility profile of \textit{E. coli} and \textit{Salmonella} spp.}

Total 76 (38%) isolates were identified as \textit{E. coli} and 46 (23%) as \textit{Salmonella} spp. after morphological, biochemical and serological characterisation. Antimicrobial susceptibility testing revealed that 68, 57, 54 and 43% \textit{E. coli} isolates were resistant to aztreonam, ceftazidime, cefotaxime and ampicillin, respectively. Among \textit{Salmonella} spp., 76 and 50% isolates were found resistant against oxytetracycline and ampicillin, respectively.

In \textit{E. coli}, highest MAR index of 0.88 was recorded for 12 isolates, followed by 0.77 for 6 isolates, 0.66 for 8 isolates, 0.55 for 10 isolates, 0.44 for 5 isolates, 0.33 for 13 isolates, 0.22 for 10 isolates and 0.11 for 5 isolates. However MAR index of 0.00 was recorded for 7 isolates. MAR index recorded for the 46 \textit{Salmonella} isolates were: 1.00 for 1 isolate, 0.88 for 5 isolates, 0.77 for 4 isolates, 0.66 for 2 isolates, 0.55 for 3 isolates, 0.44 for 6 isolates, 0.33 for 2 isolates, 0.22 for 12 isolates and 0.11 for 11 isolates. MAR index above 0.2 for the isolates indicated non-judicious use of antibiotics. Out of 76 \textit{E. coli} and 46 \textit{Salmonella} spp. isolates, 31 (40.8) and 19 (41.3%) isolates, respectively, were phenotypically identified as ESBL producers.

\textit{Molecular characterisation of ESBL genes by m-PCR}

Out of total 122 isolates, m-PCR detected at least one ESBL gene in 16 isolates (Table 2). \textit{bla\textsubscript{TEM}} was detected in a total of 10.7% (13/122) isolates whereas \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{SHV}} genes were detected in 1.6% (2/122) and 0.8% (1/122) isolates, respectively. Frequency of occurrence of these ESBL genes was higher in \textit{E. coli} as compared to \textit{Salmonella} isolates. Occurrence of \textit{bla\textsubscript{TEM}} was higher in \textit{E. coli} (13.2%) as compared to \textit{Salmonella} spp. (6.5%) isolates; however, none was detected positive for \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{SHV}} genes (Table 2). Further analysis revealed that 13 and 5.7% of ESBL genes were detected in plasmid DNA and genomic DNA, respectively.

\textit{Nucleotide sequencing analysis}

In the present study, nucleotide and protein sequences of \textit{bla\textsubscript{TEM}} gene of strain \textit{E. coli} R9 (accession no. MN052808) shared 100% similarity while \textit{bla\textsubscript{TEM}} gene of strain \textit{E. coli} R12 (accession no. MN052809) shared more than 99% similarity with all other retrieved sequences (accession nos. KY466959, KY466966, KY466969, MG653157, MG653159, MG653163). Furthermore, sequence of \textit{bla\textsubscript{TEM}} gene of strain \textit{E. coli} R9 (accession no. MN052808) revealed one variation in the \textit{bla\textsubscript{TEM}} gene from all the above retrieved sequences at position 436 (CGG → GGG). This mutation leads to change in amino acid arginine to glycine. Further, both \textit{bla\textsubscript{CTX-M}} sequences of \textit{Salmonella} (accession no. MN052811 and MN 052812) were different at two positions i.e. 9 (Ser, TCT → TCC) and 18 (Lys, AAA → AAG), moreover, sequence of strain \textit{Salmonella} R26 (accession no. MN052811) also differs

| Table 1. Details of primers used for multiplex-Polymerase Chain Reaction (m-PCR) |
|-----------------------------------------------|
| **Target gene** | **Primer sequence** | **Amplicon size (bp)** |
| \textit{bla\textsubscript{SHV}} | Forward: ATGCCGTATATTGGCCTG | 747 |
| | Reverse: TGCTTTGTTATTGGGCAA | |
| \textit{bla\textsubscript{TEM}} | Forward: TCGCGCATACATATTCAGA | 445 |
| | Reverse: ACGTCACGGCTCCAGATTAT | |
| \textit{bla\textsubscript{CTX-M}} | Forward: ATGTGCAGYACCAGTAARGTKATGC | 593 |
| | Reverse: TGGGTRAARTARGTSACCAGAAYCAGCGG | |

| Table 2. Distribution of various ESBL genes among \textit{E. coli} and \textit{Salmonella} isolates of fish |
|-----------------------------------------------|
| **Isolates (n)** | **\textit{bla\textsubscript{TEM}}** | **\textit{bla\textsubscript{CTX-M}}** | **\textit{bla\textsubscript{SHV}}** |
| \textit{E. coli} (76) | 10 (13.2) | 0 (0) | 1 (1.3) |
| \textit{Salmonella} (46) | 3 (6.5) | 2 (4.4) | 0 (0) |
| Total (122) | 13 (10.7) | 2 (1.6) | 1 (0.8) |
The present study recorded prevalence of organism of faecal contamination of water and seafood. (Kumar, 2009) and 26.7% prevalence of of 23.2% in fish samples in Cochin, India (Kumar). Inadequately cleaned and disinfected boat decks and from beach seawater throughout the year (Iyer, 2000). The results test sample in standard procedures for examining sea consumption. Salmonellae should be absent in 25 g of food ready for consumption (FDA, 2011). The results of present study showed that fishes were contaminated with Salmonella spp. thus raising serious public health concern. Antimicrobial resistant pattern suggest that E. coli isolates carrying resistance genes are present in fish samples and can be a reservoir of transferable resistance genes among pathogens. The constant usage of third generation cephalosporins in aquaculture is one the reason for such incidence in the study area. A wide range of antibiotics extensively used in aquaculture develop resistant organisms and with the consumption of such contaminated fish products, these resistant strains are transferred to human gut and lead to the emergence of resistant E. coli strains to higher generation antimicrobials (Elhadi and Alsamman, 2015). The presence of resistant E. coli strains in fish may become a very serious public health concern among the seafood consumers.

In our study, maximum number of Salmonella isolates were resistant to oxytetracycline (76%) and ampicillin (50%), suggest the wide spread use of these antibiotics in fish farming. It is noticed in human and veterinary medicine that the antimicrobial resistant Salmonella is associated with extensive use of antimicrobial drugs. Antimicrobial agents are widely used for disease prevention and as a growth promoter in animal husbandry and also in fish culture. The extensive usage of the antimicrobial drugs ended in the development of resistant microbes in human, animals and environment. This increase of antimicrobial-resistant Salmonella could limit the therapeutic options for clinical cases that require antimicrobial treatment (Ruiz et al., 1999)

A variety of antibiotics have been used to treat infection caused by E. coli and Salmonella spp. and have proved useful in many cases, but sometimes these microorganisms also develop multiple antibiotic resistances. Many strains are known to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species to establish multiple antibiotic resistances (Partridge et al., 2018). The prolonged use of antibiotics has been identified as a major factor responsible for the increased incidence of antibiotics resistance (WHO, 2020).

The occurrence of ESBL producing E. coli in fish may be due to post-harvest contamination such as infected handlers, uncleaned vessels and repeated use of contaminated water in the fishery outlets. The findings of the present study indicate that multidrug-resistant Salmonella are widely disseminated in seafood, within the food distribution system. The manifestation of these multidrug-resistant strains is a public health concern and demonstrates that the use of antimicrobial agents in both humans and animals should be strictly controlled.
of genes responsible for ESBL production. The ESBL expression in bacteria is controlled by multiple genes encoded on both chromosomal and plasmid DNA. Thus multiplex PCR was used for detection of two or more genes simultaneously in a single isolate responsible for production of ESBLs. It is a matter of concern that most ESBL-producing strains are frequently cross-resistant to other classes of antimicrobial agents. This is due to the fact that bla ESBL genes are commonly located on conjugative plasmids which also harbour genes conferring resistance to other antibiotic classes such as quinolones and aminoglycosides (Zurfluh et al., 2013). 

Nucleotide sequence analyses provide the actual pattern of nucleotides in a particular fragment of DNA and enable the confirmation and accurate identification of specific genes. PCR amplification with gene sequencing is considered as the gold standard for final characterisation and confirmation of antimicrobial resistance genes in bacteria (Teo et al., 2012). In the present study, both bla<sub>TEM</sub> sequences were identified as bla<sub>TEM-116</sub>, which was detected for the first time in Korea, Spain and Uruguay in Klebsiella pneumoniae and E. coli strains, is different from bla<sub>TEM-1</sub> in two mutation points (Hu et al., 2008). It was further detected in Salmonella and Providencia stuartii strains from South Africa and Tunisia, respectively (Usha et al., 2008; Lahlauoui et al., 2011). The bla<sub>TEM-116</sub> is an extended spectrum β-lactamase with preferential hydrolytic activity to cefazidime and cefotaxime antibiotics (Jeong et al., 2004). Furthermore, both obtained bla<sub>CTX-M-15</sub> sequences in the present study were identified as bla<sub>CTX-M-15</sub>, which belongs to the bla<sub>CTX-M</sub> group 1, suggesting the circulation of such strains in environment. The bla<sub>CTX-M-15</sub> is often reported in members of Enterobacteriaceae family from many countries like the UK, Turkey, Spain, Norway, Italy, Portugal (Coque et al., 2008), Korea (Song et al., 2009) and also from India (Haque et al., 2012). The bla<sub>CTX-M-15</sub> differs from bla<sub>CTX-M-1</sub> by an Asp-240 → Gly substitution that increases activity against cefazidime (Poirel et al., 2002). The bla<sub>CTX-M-11</sub> enzyme is increasingly detected in multidrug-resistant strains showing resistance to at least two of the following antibiotics: fluoroquinolones, tetracycline and aminoglycosides (Pitout et al., 2005). To the best of our knowledge, this study reports the first detection of bla<sub>TEM-116</sub> and bla<sub>CTX-M-15</sub> in foodborne pathogens of fish origin in India. ESBLs are considered to be one of the most important antibiotic resistance mechanisms. This study illustrates that fish may serve as important reservoir for antibiotic resistance genes carrying foodborne pathogens in the environment and might serve as vector for transmission of such genes to human commensal organisms and pathogens of clinical importance. Therefore, in order to minimise the spread of these most important ESBL-producing antibiotic resistance strains, antimicrobials should be used judiciously in fish farming.

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