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Prevalence and molecular characterisation of extended-spectrum β-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 β-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* spp., 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}, andbla_{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 β-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 β-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 tetrathionate 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), ceftazidime (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_{TEM}, bla_{SHV} and bla_{CTX-M} genes by multiplex-polymerase chain reaction (m-PCR) following the protocol given by Monstein *et al*. (2007). The oligonucleotide primers specific for the bla_{SHV} (Paterson *et al*., 2005), bla_{TEM} (Apaka *et al*., 2010) and bla_{CTX-M} (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 $MgCl_2$), 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 electrophorosed 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_{TEM} genes

Table 1. Details of primers used for multiplex-Polymerase Chain Reaction (m-PCR)

Target gene	Primer sequence	Amplicon size (bp)
bla_{SHV}	Forward: ATGCGTTATATTCGCCTGTG Reverse: TGCTTTGTTATTCGGGCCAA	747
bla_{TEM}	Forward: TCGCCGCATACACTATTCTCAGA Reverse: ACGCTCACCGGCTCCAGATTTAT	445
bla_{CTX-M}	Forward: ATGTGCAGYACCAGTAARGTKATGGC Reverse: TGGGTRAARTARGTSACCAGAAYCAGCGG	593

of *E. coli* and two bla_{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 bla_{TEM} gene of strain *E. coli* R9 and *E. coli* R12 were submitted to GenBank under the accession numbers MN052808 and MN052809, respectively. Similarly, the partial CDS of bla_{CTX-M} gene of strain *Salmonella* R26 and *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

Isolation, identification and antimicrobial susceptibility profile of E. coli and Salmonella spp.

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

In *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 *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 *E. coli* and 46 *Salmonella* spp. isolates, 31 (40.8) and 19 (41.3%) isolates, respectively, were phenotypically identified as ESBL producers.

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

Nucleotide sequencing analysis

In the present study, nucleotide and protein sequences of bla_{TEM} gene of strain *E. coli* R9 (accession no. MN052808) shared 100% similarity while bla_{TEM} gene of strain *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 bla_{TEM} gene of strain *E. coli* R9 (accession no. MN052808) revealed one variation in the bla_{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 bla_{CTX-M} sequences of *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 *Salmonella* R26 (accession no. MN052811) also differs

Table 2. Distribution of various ESBL genes among *E. coli* and *Salmonella* isolates of fish

Isolates (n)	ESBL genes present in number of isolates [n (%)]		
	bla_{TEM}	bla_{CTX-M}	bla_{SHV}
E. coli (76)	10 (13.2)	0 (0)	1 (1.3)
Salmonella (46)	3 (6.5)	2 (4.4)	0 (0)
Total (122)	13 (10.7)	2 (1.6)	1 (0.8)

at position 582 (Thr, ACT → ACC) but all these three changes are synonymous with all other retrieved sequences (accession nos. AB751242, FJ654733, HM117627, HQ256746 and MF797871). Further, phylogenetic tree was constructed on the basis of nucleotide as well as deduced amino acid sequences with the different retrieved bacterial strains. Phylogram revealed that both bla_{TEM} sequences obtained are of $bla_{TEM-116}$ type. In case of bla_{CTX-M} gene, both sequences obtained were identified as $bla_{CTX-M-15}$ variants. The bla_{TEM} sequence of strain *E. coli* R9 (accession no. MN052808) belongs to same clade with other retrieved sequences but bla_{TEM} sequence of strain *E. coli* R12 (accession no. MN052809) revealed the dissemblance and belongs to new sub clade. Both bla_{CTX-M} sequences of *Salmonella* obtained (accession no. MN051811 and MN052812) belong to same clade and it also shared sub clade with sequence MF797871.

Discussion

Present study recorded 38% prevalence of *E. coli* in fish samples while on contrary higher prevalence (65%) in Kolkata (Dutta *et al*., 2015) and 66% in retail fish samples in Cochin (Thampuran *et al.*, 2005) have been reported. However, lower prevalence of 18% was reported in Iran (Tajbakhsh *et al.*, 2015). The present study indicates that *E. coli* contamination of tropical seafood is quite common. In India, *E. coli* has been isolated from beach seawater throughout the year (Iyer, 2000). Inadequately cleaned and disinfected boat decks and fish containers are known to contaminate the catch with *E. coli.* Contamination can also occur from ice, unclean workers and unhygienic handling after catching of fish and shellfishes (Iyer, 2000). *E. coli* act as an indicator organism of faecal contamination of water and seafood. The present study recorded prevalence of *E. coli* in samples of fish and shellfish indicating poor hygienic and sanitary quality of fish.

In the present study, 23.8% prevalence of *Salmonella* in fish samples was observed. Similar prevalence rate of 23.2% in fish samples in Cochin, India (Kumar *et al.*, 2009) and 26.7% prevalence of *Salmonella* serotype *S. derby* in 150 fish samples in Egypt (Bakr *et al*., 2011) were also reported. However, lower prevalence rate of 20% of *Salmonella* have been reported from Malaysia (Sing *et al*., 2016). The significantly higher prevalence of 49.6% was reported in fish samples from Kenya (Onyuka *et al*., 2011). *Salmonella* is considered as most common food borne pathogen throughout the globe. The *Salmonellosis* incidence is linked to the intake of fish and fishery products similar to the case of meat consumption. Salmonellae should be absent in 25 g of test sample in standard procedures for examining sea 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 resistance 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 use 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. The genotypic methods help us to confirm the presence

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_{TEM} sequences were identified as $bla_{TEM-116}$ which was detected for the first time in Korea, Spain and Uruguay in *Klebsiella pneumoniae* and *E. coli* strains, is different from bla_{TEM-1} 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; Lahlaoui *et al*., 2011). The $bla_{TEM-116}$ is an extended spectrum β-lactamase with preferential hydrolytic activity to ceftazidime and cefotaxime antibiotics (Jeong *et al*., 2004). Furthermore, both obtained bla_{CTX-M} sequences in the present study were identified as $bla_{CTX-M-15}$, which belongs to the bla_{CTX-M} group 1, suggesting the circulation of such strains in environment. The $bla_{CTX-M-15}$ 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 $bla_{CTX-M-15}$ differs from $bla_{CTX-M-3}$ by an Asp-240 → Gly substitution that increases activity against ceftazidime (Poirel *et al*., 2002). The $bla_{CTX-M-15}$ 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 $bla_{TEM-116}$ and $bla_{CTX-M-15}$ 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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