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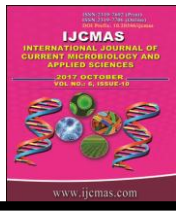

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Detection of Beta-Lactam Resistance in Piscean *Escherichia coli* using Combination Disc Method and Multiplex PCR

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A B S T R A C T

Intestinal contents from freshwater fish (*Catla catla*) (n=150) were bacteriological cultured for *Escherichia coli* and confirmed by PCR targeting *E16S* gene. A total of 104 *E. coli* isolates were recovered and analyzed for the presence of extended-spectrum beta-lactamases (ESBLs), phenotypically by disc diffusion method and genotypically by multiplex PCR targeting bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M} and bla_{AmpC} beta-lactamase (*bla*) genes. Phenotypic beta-lactam resistance was detected in a total of 16 isolates, of which nine isolates carried ESBL phenotype by combination disc method. Multiplex PCR assay revealed presence of bla_{AmpC}, bla_{TEM}, bla_{CTX-M}, bla_{SHV} and bla_{OXA} genes in 13, 13, 9, 6 and 4 isolates, respectively. Serotyping of beta-lactam resistant *E. coli* isolates (n=16) revealed O120 (2 isolates), O141 (2), rough (2), O63 (1), O126 (1) and untypable (8) serotypes. This was the first report on the phenotypic detection and molecular confirmation of ESBL producing *E. coli* from freshwater fish in Andhra Pradesh.

Introduction

Escherichia coli is a normal inhabitant of intestinal tract of warm-blooded animals (Kaper *et al.*, 2004). Owing to contamination of water bodies with human and animal excreta, *E. coli* is considered as one of the most important food borne pathogen in fish and fish products (Costa, 2013). The microbiological quality of unprocessed fish as well as the antibiotic susceptibility patterns of food borne pathogens strongly determines the quality and public health significance of fish products (Gram, 1992). The indiscriminate use of antibiotics and improper disposal into the environment has lead to the selection and dissemination of antibiotic-resistant strains (Pfeifer *et al.*, 2010). Resistance to beta-lactam antibiotics is mediated by bacterial enzymes called beta-lactamases that are encoded by beta-lactamase (*bla*) genes like bla_{TEM} (Temoniera β-lactamase), bla_{SHV} (sulfhydryl variable), bla_{OXA} (oxacillinase), bla_{CTX-M} (Cefotaximase-Munich), bla_{AmpC} etc (Bush and Jacoby, 2010). Extended-spectrum beta-lactamases (ESBLs) are variants of beta-lactamases that confer resistance to third generation cephalosporins as well as monobactams and are inhibited by beta-lactamase inhibitors (Bush and Jacoby, 2010).

Studies related to the detection of beta-lactam resistance in *Escherichia coli* of fish origin have been relatively less explored in India. The present study was undertaken with an objective of phenotypic and molecular detection of beta-lactam antimicrobial resistance in *E. coli* isolated from freshwater fish in Andhra Pradesh.

Materials and Methods

Bacterial reference strains and primers

The reference strains, beta-lactamase negative *E. coli* (ATCC 25922) and beta-lactamase positive *Klebsiella pneumoniae* (ATCC 700603) were procured from M/s. HiMedia Laboratories (Mumbai). Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample collection

A total of 150 freshwater fish intestinal samples (10g each) were collected from fish markets (Andhra Pradesh, India) in sterile polythene zip lock packs and immediately transported on ice to the food safety laboratory at the Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science (Gannavaram, Andhra Pradesh).

Isolation and PCR confirmation of *E. coli*

Samples were homogenized and inoculated into nutrient broth and incubated aerobically at 37°C for 24 h. Loopful of enriched broth was streaked onto eosin methylene blue (EMB) agar, incubated at 37ºC for 24 h. Green metallic sheen colonies were picked up onto nutrient agar slants as pure culture and subjected to standard biochemical tests (Sneath and Holt, 2001). Whole cell DNA extraction was carried out by boiling and snap chilling method (Sekhar *et al.*, 2017). PCR confirmation of *E. coli* was done using oligonucleotide primers (F, 5'- ATC AAC CGA GAT TCC CCC AGT-3' and R, 5'- TCA CTA TCG GTC AGT CAG GAG-3') targeting the *E16S* gene of *E. coli* (Sharif *et al.*, 2017).

Detection of ESBL production

E. coli isolates were screened for resistance against third generation cephalosporins like cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and monobactams like aztreonam (30 µg) by disc diffusion method (Bauer *et al.*, 1966) on Mueller-Hinton agar using commercial discs (HiMedia). Resistance to at least one of the antibiotics used was considered as positive screening test for ESBL production (Drieux *et al.*, 2008 and CLSI, 2014). Isolates that were found positive in the screening test were further subjected to ESBL confirmatory test by combination disc method (CDM), where the inhibition zone around a disc of cephalosporin alone and around a disc of the same cephalosporin plus clavulanate/sulbactam was measured i.e. ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg). A difference of $\geq$ 5 mm between the two diameters indicates ESBL production (Drieux *et al.*, 2008 and CLSI, 2014).

Detection of beta-lactamase genes

Phenotypically resistant *E. coli* isolates were subjected to PCR for the detection of beta-lactamase genes as described by Dallenne *et al.*, (2010) and Sharif *et al.*, (2017) with slight modifications. For the detection of bla_{TEM}, bla_{SHV} and bla_{OXA} genes, multiplex PCR (Table 1) was carried out in 25 µl reaction volume containing 2 µl of DNA template

prepared from each isolate; *Taq* buffer (10x) - 3 µl; dNTP mix (10mM) - 1 µl; $MgCl_2$ (25mM) - 1.5 µl; three forward primers (10 pmol/µl) - each 0.5 µl; three reverse primers (10 pmol/µl) - each 0.5 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water - 13.5 µl. For the detection of bla_{CTX-M} gene, PCR (Table 1) was carried out in 25 µl reaction volume containing 1.5 µl of DNA template; *Taq* buffer (10x) – 2.75 µl; dNTP mix (10mM) – 0.5 µl; $MgCl_2$(25mM) - 1 µl; forward primer (10 pmol/µl) – 1.5 µl; two reverse primer (10 pmol/µl) – 1.5 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water – 15.25 µl. Both the PCR assays were carried out in Eppendorf thermal cycler under standardized cycling conditions - initial denaturation at 94ºC for 10 min, 30 cycles of denaturation at 94ºC for 40 sec, annealing at 60ºC for 40 sec, elongation at 72ºC for 1 min, final elongation at 72 ºC for 7 min and hold at 4ºC.

For the detection of bla_{AmpC} gene, PCR (Table 1) was optimized in 25 µl reaction mixture containing 1 µl of DNA template; *Taq* buffer [10x] – 2.5 µl; dNTP mix [10mM] – 0.5 µl; $MgCl_2$[25mM] - 1.5 µl; forward primer [10 pmol/µl] - 1 µl; reverse primer [10 pmol/µl] - 1 µl; *Taq* DNA polymerase [1 U/µl] - 1 µl and nuclease free water – 16.5 µl, under standardized cycling conditions: initial denaturation of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Final extension was done at 72°C for 10 min. Known positive DNA was used as positive control in all the PCR reactions.

Serotyping of beta-lactam resistant *E. coli* isolates

Serotyping of beta-lactam resistant *E. coli* isolates on the basis of their 'O' antigen was performed at National *Salmonella* and *Escherichia coli* Centre (NSEC), Central Research Institute (CRI), Kasauli, Himachal Pradesh, India.

Results and Discussion

Out of 150 samples analyzed, *E. coli* was isolated from 104 (69.3%) samples. In a study from Punjab, Gupta *et al.,* (2003) observed isolation of *E. coli* from 48.9% of raw fish samples. All the biochemically characterized *E. coli* isolates recovered in the present study amplified 231 bp product in the PCR targeting *E16S* gene of *E. coli*. The PCR targeting *E16S* gene was successfully used earlier by other workers for the molecular confirmation of *E. coli* isolated from diverse sources (Ahmadi *et al.,* 2015 and Sharif *et al.,* 2017).

Investigating the level of antibiotic resistance among gut commensals such as *E. coli* is considered as a good indicator of prevalence and spread of antimicrobial resistance (EFSA, 2013). Sixteen out of 104 *E. coli* isolates screened were found to be resistant to one or more of the indicator antibiotics, with an overall incidence of 15.3% beta-lactam resistance (Table 2).

Overall frequency of resistance to cefotaxime, ceftriaxone, ceftazidime and aztreonam was found to be 12.5 (13/104), 11.5 (12/104), 6.7 (7/104) and 5.7% (6/104), respectively. Our findings are in contrast with those of Helba (2013) and Carvalho *et al.,* (2016), who reported complete ceftriaxone and cefotaxime sensitivity in *E. coli* isolates recovered from fish. Contrast to this, Saqr *et al.,* (2016) observed higher resistance (67.5%) to cefotaxime in Piscean *E. coli*. Out of 16 *E. coli* isolates that were found positive in screening test, ESBL production was confirmed in nine isolates by combination disc method, giving an overall frequency of 8.6% (9/104). In the remaining seven isolates, enhancement of inhibition zone by ≥5 mm in the presence of clavulanic acid/sulbactam was not observed. This might be due to concurrent

production of other non-ESBL beta-lactamases that were resistant to beta-lactamase inhibitors, masking the synergy in the confirmatory test (Drieux *et al.*, 2008). Compared to the present study, relatively higher incidence of ESBL phenotype (62.5%) was observed by Le *et al.*, (2015) in *E. coli* of fish origin.

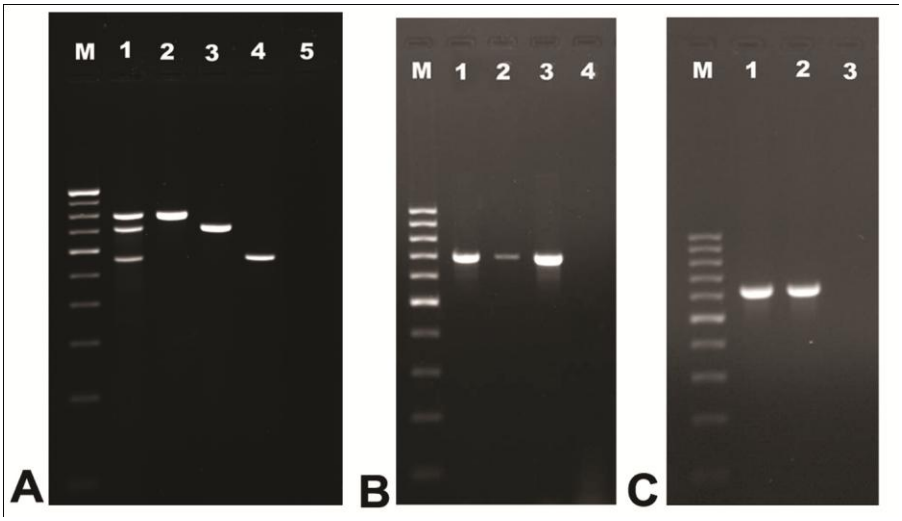
Table.1 Oligonucleotide primers used for the detection of beta-lactamase genes

Primer	Primer sequence (5'-3')	Amplicon size
Multiplex PCR for the detection of TEM, SHV and OXA genes		
bla$_{TEM}$	F: CATTTCCGTGTCGCCCTTATTC	800 bp
	R: CGTTCATCCATAGTTGCCTGAC	
bla$_{SHV}$	F: AGCCGCTTGAGCAAATTAAAC	713 bp
	R: ATCCCGCAGATAAATCACCAC	
bla$_{OXA}$	F: GGCACCAGATTCAACTTTCAAG	564 bp
	R: GACCCCAAGTTTCCTGTAAGTG	
PCR for the detection of CTX-M gene		
bla$_{CTX-M}$	F: TTAGGAAATGTGCCGCTGTA	688 bp
	R: CGATATCGTTGGTGGTACCAT	
PCR for the detection of AmpC gene		
bla$_{AmpC}$	F: CCCCGCTTATAGAGCAACAA	631 bp
	R: TCAATGGTCGACTTCACACC	

Table.2 Beta-lactam resistant phenotypes and genotypes of Piscean *E. coli*

S. No	Serotype	Resistance to beta-lactam antibiotics in screening test				ESBL phenotypic confirmation	Beta-lactamase genes detected
		CTX	CTR	CAZ	AT		
1.	O120	R	R	S	R	positive	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
2.	O120	R	R	S	R	positive	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
3.	O141	R	S	R	S	-	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{AmpC}$
4.	O141	R	R	R	S	positive	*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
5.	O63	R	R	S	S	positive	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
6.	O126	S	R	S	S	-	*bla*$_{TEM}$, *bla*$_{AmpC}$
7.	Rough	R	S	S	R	positive	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
8.	Rough	R	R	R	R	positive	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
9.	UT	R	R	S	S	-	-
10.	UT	R	R	R	R	positive	*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
11.	UT	S	R	S	S	-	-
12.	UT	R	R	S	R	positive	*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$,
13.	UT	R	S	R	S	-	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{AmpC}$
14.	UT	R	S	R	S	-	-
15.	UT	S	R	S	S	-	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{AmpC}$
16.	UT	R	R	R	S	positive	*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{AmpC}$
TOTAL (16)		**13**	**12**	**7**	**6**	**9**	

Fig.1 (A). Agarose gel electrophoresis of *bla*$_{TEM,}$ *bla*$_{SHV}$ and *bla*$_{OXA}$ gene amplicons of *Escherichia coli.* Lane M: 100 bp DNA ladder, L1: DNA standard carrying *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes, L2: *bla*$_{TEM}$ gene (800 bp), L3: *bla*$_{SHV}$ gene (713 bp), L4: *bla*$_{OXA}$ gene (564 bp) and L5: Negative control (B). Agarose gel electrophoresis of *bla*$_{CTX-M}$ gene amplicons. L1: DNA standard for *bla*$_{CTX-M}$ gene, L2-3: *bla*$_{CTX-M}$ gene (688 bp), L4: Negative control (C). Agarose gel electrophoresis of *bla*$_{AmpC}$ gene amplicons. L1: DNA standard for *bla*$_{AmpC}$ gene, L2: *bla*$_{AmpC}$ gene (631 bp) and L3: Negative control



Among the beta-lactam resistant isolates (n=16), one or more beta-lactamase genes were detected in a total of 13 isolates (Table 2), whereas no beta-lactamase genes were detected in three isolates. O'Keefe *et al.,* (2010) and Hordijk *et al.,* (2013) also failed to detect beta-lactamase genes in few *E. coli* isolates with beta-lactam resistant phenotype. Several explanations had been put forward by many workers for the possible expression of resistant phenotype in the absence of beta-lactamase genes (Drieux *et al.,* 2008). One explanation could be the contribution of other resistance mechanisms, such as enhanced expression of efflux pumps (O'Keefe *et al.,* 2010 and Hordijk *et al.,* 2013).

Beta-lactamase genes detected in the present study were as follows - *bla*$_{AmpC}$, *bla*$_{TEM}$, *bla*$_{CTX-M,}$ *bla*$_{SHV}$ and *bla*$_{OXA}$ genes in 13, 13, 9, 6 and 4 isolates, respectively (Figure 1). Beta-lactamase genes were detected in all the 13 *E. coli* isolates with 'ESBL' phenotype. Van *et al.,* (2008) and Ryu *et al.,* (2012) reported detection of beta-lactamase (blaTEM) genes in *E. coli* isolated from commercial fish, whereas failed to detect other beta-lactamase genes like SHV, OXA and AmpC. Le *et al.,* (2015) observed multi resistance genes (CTX-M and TEM) in about 50% of the *E. coli* isolates recovered from fish in Vietnam.

Serological typing of beta-lactam resistant isolates (n=16) revealed O120 (2 isolates), O141 (2), rough (2), O63 (1), O126 (1) and untypable (8) serotypes (Table 2). Gupta *et al.,* (2013) reported isolation of O5, O11, O17, O28, O41, O58, O69, O103, O168 and O170 serotypes of *E. coli* from raw fish in a study from Punjab (India). Rao (2009) reported O-serogroups O20, O17, O53, O78, O86, O22, O24, O46, O110 and O153 among *E. coli* isolated from fish. Among the *E. coli* serotypes detected in the present study, O126 serotype was reported to be pathogenic to humans (Nataro and Kaper, 1998).

The present study highlighted the occurrence of ESBL antimicrobial resistance in *E. coli* isolated from freshwater fish in Andhra Pradesh, which may pose threat to consumers of fish and fish products. There is a need of thorough control over microbiological quality of fish in order to safe guard the public health. In addition, the present study also signifies the need for a comprehensive antimicrobial surveillance programme to determine the prevalence of ESBL resistance among various fish borne pathogens of public health significance.

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