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

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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 blaTEM (Temoniera β-lactamase), blaSHV (sulphhydryl variable), blaOXA (oxacillinase), blaCTX-M (Cefotaximase-Munich), blaAmpC 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 37°C 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 16S gene of *E. coli* (Sharif et al., 2017).

#### Detection of ESBL production

*E. coli* isolates were screened for resistance against third generation cephalosporins like ceftazidime (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 ≥ 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<sub>TEM</sub>, bla<sub>SHV</sub> and bla<sub>OXA</sub> 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 94°C for 10 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, elongation at 72°C for 1 min, final elongation at 72 °C for 7 min and hold at 4°C.

For the detection of *bla*A$_{mpC}$ 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 ceftaxime, 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 ceftaxime sensitivity in *E. coli* isolates recovered from fish. Contrast to this, Saqr et al., (2016) observed higher resistance (67.5%) to ceftaxime 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/sublactam 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 |
|---------------|-------------------------|---------------|
| *bla*<sub>TEM</sub> | F: CATTTCCTGTCGCCCTTATTTC<br>R: CTTTCATCCATAGGCTGCTGAC | 800 bp |
| *bla*<sub>SHV</sub> | F: AGCCCGCTTGAACATTAAAC<br>R: ATCCCGCAATAAAATCACAGC | 713 bp |
| *bla*<sub>OXA</sub> | F: GGCACAGATTCAACTTCAAG<br>R: GACCCCAAGTTTCCTGAAGTG | 564 bp |
| **PCR for the detection of CTX-M gene** | | |
| *bla*<sub>CTX-M</sub> | F: TTAGGAAATGTCGGCCCTGTA<br>R: CGATATCGCTGTGCTACCAT | 688 bp |
| **PCR for the detection of AmpC gene** | | |
| *bla*<sub>AmpC</sub> | F: CCCCCTTATAGAGCAACAA<br>R: TCAATGGTGGCTACCAC | 631 bp |

**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*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 2.    | O120     | R   | R   | S   | R  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 3.    | O141     | R   | S   | R   | S  | -                      | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>AmpC</sub> |
| 4.    | O141     | R   | R   | R   | S  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 5.    | O63      | R   | R   | S   | S  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 6.    | O126     | S   | R   | S   | S  | -                      | *bla*<sub>TEM</sub>, *bla*<sub>AmpC</sub> |
| 7.    | Rough    | R   | S   | S   | R  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 8.    | Rough    | R   | R   | R   | R  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 9.    | UT       | R   | R   | S   | S  | -                      | -                             |
| 10.   | UT       | R   | R   | R   | R  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 11.   | UT       | S   | R   | S   | S  | -                      | -                             |
| 12.   | UT       | R   | R   | S   | R  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 13.   | UT       | R   | S   | R   | S  | -                      | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>AmpC</sub> |
| 14.   | UT       | R   | S   | R   | S  | -                      | -                             |
| 15.   | UT       | S   | R   | S   | S  | -                      | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| 16.   | UT       | R   | R   | R   | S  | positive              | *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>AmpC</sub> |
| TOTAL (16) |          | 13  | 12  | 7   | 6  | 9                       |                               |
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\textsubscript{AmpC}, bla\textsubscript{TEM}, bla\textsubscript{CTX-M}, bla\textsubscript{SHV} and bla\textsubscript{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 (bla\textsubscript{TEM}) 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.

Acknowledgements

The authors acknowledge Sri Venkateswara Veterinary University (SVVU), Tirupati, Andhra Pradesh for supporting the research work and National Salmonella and Escherichia Centre (NSEC), Central Research Institute (CRI), Kasauli, Himachal Pradesh (India), for serotyping of E. coli isolates.

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**How to cite this article:**

Soma Sekhar, M., N. Mohammad Sharif, T. Srinivasa Rao and Bindu Kiranmayi, Ch. 2017. Detection of Beta-Lactam Resistance in Piscine *Escherichia coli* using Combination Disc Method and Multiplex PCR. *Int.J.Curr.Microbiol.App.Sci.* 6(10): 209-215.
doi: [https://doi.org/10.20546/ijcemas.2017.610.026](https://doi.org/10.20546/ijcemas.2017.610.026)