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Short Communication

Phenotypic and Genotypic Analysis of Beta-Lactamases in *Escherichia coli* Isolated from Fish

Kafeel Ahmad*, Muhammad Siqaf, Gul-E-Rana Abid, Ramla Somayya and Umama Qasim

Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Pakistan

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KA conceived the idea, contributed to experimental work, conducted analysis, contributed to manuscript writing and proofreading. MS and GA conducted the experimental work and analysis and wrote the manuscript. RS and UQ contributed to analysis and proofreading. All the authors approved the final version of the manuscript.

Key words
Escherichia coli*, ESBL, MBL, *blaCTX-M, blaSHV, blaOXA-10

ABSTRACT

Escherichia coli is a member of Enterobacteriaceae that causes gastrointestinal diseases. The aim of current study was to analyse production of extended spectrum beta lactamases and metallo beta-lactamases in *E. coli* isolated from fish. Fifty *E. coli* isolates recovered from fish samples were screened phenotypically for extended spectrum beta lactamases and metallo beta-lactamases production by disc diffusion method using the antibiotics cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), amoxicillin-clavulanic acid (30 µg), imipenem (10 µg) and meropenem (10 µg). Three extended spectrum beta lactamases genes (*blaCTX-M*, *blaSHV* and *blaOXA-10*) and one metallo beta-lactamase gene (*blaNDM-1*) were analysed in the isolates. Eight (16%) isolates were positive for extended spectrum beta-lactamases production phenotypically. However, all isolates were negative for metallo beta-lactamases production phenotypically. The prevalence of *blaCTX-M* was 40% (n=20) and prevalence of *blaSHV* was 60% (n=30) while *blaOXA-10* was not detected. The metallo beta-lactamase gene *blaNDM-1* was detected in 6% (n=3) isolates. The high prevalence of beta-lactamases in *E. coli* isolated from fish raises serious health safety questions as these bacteria could transfer resistance genes to other pathogenic bacteria through horizontal and vertical gene transfer which could make them superbugs and non-curable through antibiotics. Hence, proper hygienic conditions should be maintained in order to stop the spread of these bacteria to human population.

Fish is a good source of vitamins, minerals and protein in human diet around the world (Costa, 2013). Due to bacterial infections in the aquaculture settings, the fish industry sometimes suffers from huge economical losses and produce low quality fish. Mostly fish is affected by members of Enterobacteriaceae such as *Escherichia coli* in the aquaculture system. These bacteria mostly inhabit contaminated water, or they are found in the body of apparently normal fish (Rocha *et al*., 2014). *E. coli* is Gram-negative rod-shaped bacterium that is present in water, soil and in the gastrointestinal tract of humans and other animals. They are normally not harmful, but some virulent strains could cause diseases. Five major pathogenic groups of *E. coli* have been identified named as Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enterohemorrhagic (EHEC), Enteroinvasive (EIEC), and Enteroaggregative *E. coli*. In order to reduce infections by these bacteria in fish and to promote growth, antibiotics have been largely used in aquaculture (Alderman and Hastings, 1998). This excess use of antibiotics has led to the development of large reservoir of antibiotic resistant *E. coli* in aquatic niche. Antibiotic-resistant genes could be transferred from non-harmful *E. coli* to the disease-causing pathogenic bacteria. This phenomenon of antibiotics-resistance could limit the action of antibiotics resulting in major loss to this fish sector (Rocha *et al*., 2014). Several antibiotic resistance genes have been reported in *E. coli* including beta-lactamases (blaTEM, *blaCTX-M*), tetracycline resistance genes (*tetA*), and genes for aminoglycoside resistance (*aadA2*) (Hussain *et al*., 2012). Extended Spectrum Beta Lactamase (ESBL) genes have previously been reported to confer antimicrobial resistance in case of fish diseases (Shaikh *et al*., 2015). ESBL gene named *blaCTX-M* is supposed to cause antimicrobial resistance in most of Gram negative bacteria threatening the application of modern antibiotics (Hernandez *et al*., 2013). ESBL producing *E. coli* may spread to humans from farm or pound fish and animals through the food chain (Rocha *et al*., 2014; Börjesson *et al*., 2016; Katakweba *et al*., 2015; Nguyen *et al*., 2016). These genes are transferred by direct or indirect mechanisms from one to another ecosystem (Founou *et al*., 2016). *E. coli* could be transferred from fish

* Corresponding author: kafeelpbg@gmail.com
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and other food producing animals to human through food chain (Nguyen *et al*., 2016). To our knowledge, no reports are available on the molecular detection of beta lactamases in *E. coli* isolated from fish in Pakistan. The present study for the first time reports the occurrence of three ESBL genes (*blaCTX-M, blaSHV* and *blaOXA-10*) and one MBL gene called New Delhi Metallo-beta-lactamase (*blaNDM-1*) in *E. coli* of fish origin in Peshawar Pakistan.

Materials and methods

E. coli isolates were recovered from fish samples collected from local markets in Peshawar, Pakistan following standard protocols as described previously (De Vos, 2009). A total of fifty isolates were investigated in the study.

Phenotypic detection of ESBLs was carried using double disc synergy test. Amoxicillin-clavulanic acid disc was applied to the centre of Muller Hinton Agar (MHA) plate having bacterial lawn. The remaining discs of aztreonam, cefepime, cefotaxime and ceftazidime were applied around the central disc with 15-20 mm away. Samples were incubated overnight at 37˚C. Zone diameters were calculated and increase in zone towards the central disc was regarded as indication of ESBL production.

Metalo beta-lactamases were phenotypically analyzed by combine disc test. Two discs imipenem were placed about 25 mm apart onto MHA plate with bacterial lawn. One disc was applied with 5 µl 0.5 M EDTA (pH 8.0). the samples were incubated overnight at 37 ˚C. Inhibition zones were recorded and increase of ≥ 7 mm in inhibition zone around imipenem-EDTA was considered as positive result for MBL production.

For molecular detection of resistant genes, DNA was isolated according to the procedure of Wilson (1987). Quality was confirmed through gel electrophoresis. Three ESBL genes (*blaCTX-M*, *blaSHV*, *blaOXA-10*) and one MBL gene (*blaNDM-1*) were analyzed among the isolates using specific primers (Peerayeh *et al*., 2014; Shanthi *et al*., 2014). A 20 µl reaction mixture containing 4 µl 5x FIREPol Master Mix (Solis BioDyne, Cat. No. 04-12-00125), 1 µl each of gene specific forward and reverse primer, 1 µl DNA template and 13 µl molecular grade water was prepared. Previously reported amplification profiles were used for each gene (Peerayeh *et al*., 2014; Shanthi *et al*., 2014). The products of PCR were analysed on 1.5% agarose gel and compared with 100 bp plus DNA ladder (BIORON, Cat. No. 304105).

Results

A total of 50 *E. coli* isolates were recovered from fish samples. Among the 50 isolates, 16% (n=4) were phenotypically confirmed as ESBLs producers. MBL-producers were not detected by phenotypic analysis. Molecular analysis showed the occurrence of two ESBL genes i.e. *bla CTX-M* and *bla SHV*. While *bla OXA-10* gene was not detected. The occurrence of MBL gene *bla NDM-1* was also confirmed among the isolates. The presence of *bla CTX-M* was confirmed by amplification of a 552 bp fragment corresponding to the gene. The presence of *bla SHV* was confirmed by the amplification of a 230 bp fragment specific to the gene while existence of *bla NDM-1* was confirmed by the amplification of a gene specific fragment of 475 bp. The *bla CTX-M* gene was detected in 20 isolates (F6G, F7D, F7C, F11C, F14D, F13L, F14K, F13M, F13E, F13Q, F13F, F13D, F14Q, F13N, F14F, F7B, F6C, F9D, F7A and F11A). The *blaSHV* gene was present in 30 isolates (F14E, F13D, F9C, F14J, F14M, F14B, F14F, F13I, F14P, F13E, F7G, F9D, F6D, F6C, F14D, F13P, F14G, F14I, F13Q, F7I, F13O, F14K, F7A, F13A, F14O, F13N, F6A, F12A, F10D and F13C). The *blaNDM-1* gene was detected in 3 isolates (F14G, F14F and F11C).

Discussion

Recently, foodborne *E. coli* and other pathogenic members of the family *Enterobacteriaceae* are becoming a serious health risk as new strains are increasingly reported to possess multiple antibiotic resistance genes including ESBLs and MBLs. A study from Vietnam reported that nearly 30% of farmed fish and shrimp were contaminated with *E. coli* able to produce beta-lactamases (Nguyen *et al*., 2016). Another study from Vietnam reported 18.3% fish contamination with bacteria in local retail store (Le *et al*., 2015). In the same way in another study from Switzerland, the gut samples of fish were found to have 18.7% prevalence of ESBL or pAmpC-producing *E. coli* with two isolates having *blaSHV-12* and one isolate contained *blaCMY-2* gene (Abgottspon *et al*., 2014). In study in Saudi Arabia 51% fish samples had ESBL-producing *E. coli* (Elhadi and Aslmmann, 2015). In the current study, 16% *E. coli* isolates were phenotypically confirmed as ESBL producers. The beta lactamase gene *blaCTX-M* and its variants are the predominant with respect to geographical location. The *blaCTX-M-1* gene has been reported from different parts of European countries (Coque *et al*., 2008), and *blaCTX-M-8* and *blaCTX-M-2* have been reported from South America (Egervärn *et al*., 2014) and Japan (Kawamura *et al*., 2014). In Vietnam, *blaCTX-M-9* and *blaCTX-M-1* have been frequently reported in fish and meat samples (Nguyen *et al*., 2016). In the current *SHV-1* gene was the most prevalent (60%) while *CTX-M* gene was the second most abundant (40%) gene.

The increased prevalence of ESBL and MBL producing *E. coli* in fish could be due to overuse of

antibiotics in aquaculture and due to disposal of domestic waste into aquatic habitats. Aquaculture is a comparatively new field where antimicrobial drugs are profoundly used, either by directly adding antibiotics to the fish food or to the water in fish farms. The indirect dissemination of antibiotic resistance genes from antibiotic-resistant bacteria from fish bodies to human pathogenic bacteria like *E. coli* and *Salmonella* has been reported in *Aeromonas* isolated from fish (Heuer *et al*., 2009; Cabello, 2006). The appearance of MDR strains of *Vibrio cholerae* which was the cause of Latin American outbreak in the year 1992 was another example where the spread of multi drug resistance occurred through seafood and water (Weber *et al*., 1994). Similar reports on outbreaks of MDR strains of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* from farmed fish in Korea haven been documented (Oh *et al*., 2011). Increasing number antibiotic resistant *Salmonella enterica* in the United States and Europe has been proposed to have originated from aquaculture environment (Cabello, 2006; Briggs and Fratamico, 1999; Angulo, 2000; Yang *et al*., 2013).

The high prevalence of ESBL and MBL producing *E. coli* originating from fish shows the presence of a potential reservoir of pathogenic bacteria. These bacteria could spread to human population through food chain. Hence, proper surveillance system needs to be put in place to constantly monitor fish farming and related industries for existence of such resistant bacteria. Strict safety measures must be taken to monitor the points of contamination after harvesting the seafood to restrict the spread of multidrug resistant bacterial strains.

Statement of conflict of interest

The authors have declared no conflict of interest.

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