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Detection of Virulence Gene and Antimicrobial Resistance Pattern of *Escherichia coli* Isolated from Fresh Water Fish in and around Anand City, Gujarat, India

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A B S T R A C T

A wide variety of infections are caused by *E. coli* in human including foodborne illnesses that may range from diarrheal disease to life threating Hemorrhagic Colitis (HC), Haemolytic Uremic syndrome (HUS). In this present study total 200 market fish samples comprising 25 each of skin, muscle, gills, intestine as well as 25 each of water sample, knife swab, butchers'' hand swab and log swab were screened for the presence of *E. coli.* Among these samples, *E. coli* could be isolated and characterized biochemically as well as by PCR. PCR results indicated that out of 43 isolates, 4 (9.30%) isolates were positive for *stx1* gene and 7 (16.27%) isolates were positive for *stx2* gene while 1 (2.32%) isolate positive for both of genes. The antibiogram of all isolates revealed high degree of sensitivity to Chloramphenicol (95.34%) and gentamicin (93.02%) while high frequency of resistance were observed to ampicillin (67.44%) and streptomycin (32.35%). The presence of *E. coli* in market fish is considered a sanitary case and may represent a risk to the consumers. However, the presence of non-pathogenic *E. coli* in fish should be viewed as a public health concern since this bacterium is recognized as an indicator of fecal contamination. The present study supports the finding that fresh water fish can be regarded as critical source of pathogenic *E. coli*. This explains the need of strict monitoring and surveillance for effective measures of hygiene and sanitary practice during selling of fresh water fish.

Introduction

The fish contain higher amount of many important nutrients which are not commonly found in other food so it is best source of human diet. Seafood is a traditional human diet which is being consumed in many countries as an important part of diet which contains important nutrient which are generally not present in other foods such as long chain fatty acids (EPA and DHA) and selenium (Bell and Sergent, 2003).

The freshwater aquaculture production in India comprises about 2.36 million ha of ponds and tanks and accounts for nearly 55% of the total fish production in India. Different species of Indian carps like catla (*Catla catla*), rohu (*Labeo* rohita) and mrigal (*Cirrhinus mrigala)*) contribute between 70% and 75% of

the total freshwater fish production, while silver carp, grass carp, common carp and catfish make up 25% to 30% of the production (FAO, 2014).

In 2017, total fish production of India was 10.07 million metric tonnes. Constituting about 6.3% of the global fish production and the sector contributes to 1.1% of the GDP and 5.15% of the agricultural GDP. In India state like West Bengal, Andhra Pradesh, Gujarat and Kerala are the leading fish producing states (FAO, 2017).

The safety of fish and fish products were mostly depending on environment of fish harvested area and sanitary condition (Bell and Sergent, 2003). In the fish, bacteria was predominantly found in the tissue like gastrointestinal tract, gills, muscle, kidney and bladder (Dasgupta, 1992). Bacterial pathogens associated with fish were classified into indigenous and non-indigenous. The indigenous bacterial pathogens are those naturally living in the fish's habitat *e.g. Vibrio* species, *Aeromonas* species while *Clotridium botulinum, Listeria monocytogenes, Staphylococcus aurues, Salmonella* species, *Shigella* species, *Escherichia coli* were included into the non-indigenous (Kvenberg, 1991; Rodeick, 1991).

E. coli in fish was considered as an indicator to sewage pollution. Whenever water was contaminated with human and animal excreta it polluted the water bodies and favour the growth of microorganism. Fish and fish products also contaminated with surrounding polluted water as well as while using polluted water in their preparation (Zheng *et al.,* 2004; Sekar *et al.,* 2008; Bell and Sergent, 2003).

E. coli was first identified by Theodore Escherich in 1885, who isolate this bacteria from the faeces of neonates. *Escherichia coli* is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm blooded organisms (Tenaillon *et al.,* 2010). First time shiga toxin producing *Escherichia coli* (STEC) was reported at Mangalore, India and it represents only pathogenic group of *E. coli* having zoonotic significance (Khan *et al.,* 2002; Renter *et al.,* 2008). Shiga toxin producing *E. coli* have globally emerged as important zoonotic and diarrhoeal pathogens that cause bloody diarrhea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS) in humans (Tarr, 1996)

There is a lack of sufficient literature on the hygienic and bacteriological quality of fish used for human consumption in Anand city. This piece of work will be useful to safeguard the consumer's health. Keeping in view above facts this work is proposed to be undertaken to determine the hygienic quality of fresh water fish sold in the Anand city market.

Materials and Methods

Sample collection

Altogether 200 samples which includes skin, gills, muscles, intestine, water sample as well as swabs from butcher's hand, knife and log were collected from various fish selling retail meat markets of Anand city, Gujarat. The samples like skin, gills, intestine and muscles were collected in sterile vial under aseptic condition. Moreover, butcher's hand swab and their instruments' swabs (particularly log and knife) will be taken in sterile swab container under aseptic condition and transported to the laboratory in an icebox for further study

Enrichment of samples

Skin, muscle, Gills and intestine of fish were separated aseptically from the fish samples and about 10 g were inoculated in MacConkey

broth and incubated at 37˚C for 18 h while swab samples were directly inoculated into requisite medium.

Isolation and identification

A loop full of enriched culture was first streaked on MacConkey agar and incubated at 37˚C for 18-24 h. Lactose fermenting pink colored colonies from MacConkey agar were sub cultured on Eosin Methylene Blue (EMB) agar. The sub cultured colonies showing greenish metallic sheen were subjected for the biochemical tests namely Oxidase test, Indole production, Methyl red, Voges Proskaeur, Simon's citrate agar, H2S production etc. were done for the confirmation of *E. coli* as proposed by Edward and Ewing.

Extraction of genomic DNA

The DNA extraction of *E. coli* isolates was carried out by using boiling method. A loopful of culture was taken into sterilized micro centrifuge tube which contains 100 µl of DNAse and RNAse free milliQ water. Then samples were vortexed and heated at 95˚C for 10 minutes, cell debris was removed by centrifugation (7000 rpm, 6 minutes) and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

Detection of virulence genes by Polymerase Chain Reaction

The PCR was standardized for the detection of two genes viz. *stx1* and *stx2* following the methodology as described by Paton and Paton (1998) for detection of *stx1* and *stx2* genes with suitable modifications. A multiplex PCR targeting *stx*1 and *stx*2 genes of *E. coli* was optimized for the detection of virulence genes in 25 µl reaction volume containing 12.5 µl of 2X PCR Master mixture, 10 pmol of forward (stx1:5' ATAAATCGCCATTCGTTG ACTAC 3', stx2: 5' GGCACTGTCTGAAACTGCTCC 3') and reverse (stx1: 5' AGAACGCCCACTGA GATCATC 3', stx2: 5'TCGCCAGTTATCT GACATTCTG 3'), 3 µl of genomic DNA and nuclease free water upto 25 µl. Reaction mixture cycled 35 times in thermal cycler (Applied Biosystem, Sweden) with preheated lid (105 ˚C) under following cycling conditions. Each cycle consisting of 5 minutes of initial denaturation at 94 ˚C, 1 minutes of initial denaturation at 95˚C, 2 minutes of annealing at 65 ˚C, 1.30 minutes of extension at 72 ˚C and 7 minutes of final extension at 72 ˚C. On completion of PCR, amplified products were analyzed through 2 % agarose gel in gel documentation system (SynGene, Gene Genius BioImaging System, UK) and data were recorded photographically.

Antimicrobial resistance

The bacterial isolates will be subjected to *in vitro* antibiotic sensitivity test as per the method of Bauer *et al.,* (1966). *In vitro* antibiotic sensitivity test of the isolates was conducted by paper disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India).

The antibiotics discs used to test the isolates were Ampicillin ((10 µg), Amikacin (10 µg), Ciprofloxacin (10 µg), Chloramphenicol (10 µg), Gentamicin (10 µg), Enrofloxacin (10 µg), Ofloxacin (05 µg), Oxytetracycline (30 µg), Trimethoprime (30 µg), Streptomycin (25 µg). *E. coli* isolates were grown in Brain Heart Infusion (BHI) broth (HiMedia) for 12-18 hours. The grown cultures were swabbed on Muller-Hinton agar plates (HiMedia Pvt. Ltd.) with sterile cotton swabs and left for 30 minutes for pre-diffusion time. Then using an ethanol dipped and flamed forceps different antibiotic discs were placed on the agar surface about two cm apart. The discs were slightly pressed with the forceps to make complete contact with the medium. The plates

were incubated at 37°C for 18-24 hours. After the incubation period, the diameter of inhibition zones were measured and compared with interpretative chart provided by the manufacturer and zones were graded as sensitive, intermediate and resistant

Results and Discussion

The bacteriological examination of market fish samples (n=200) revealed the presence of *E coli* in 43 (22.5%) samples.

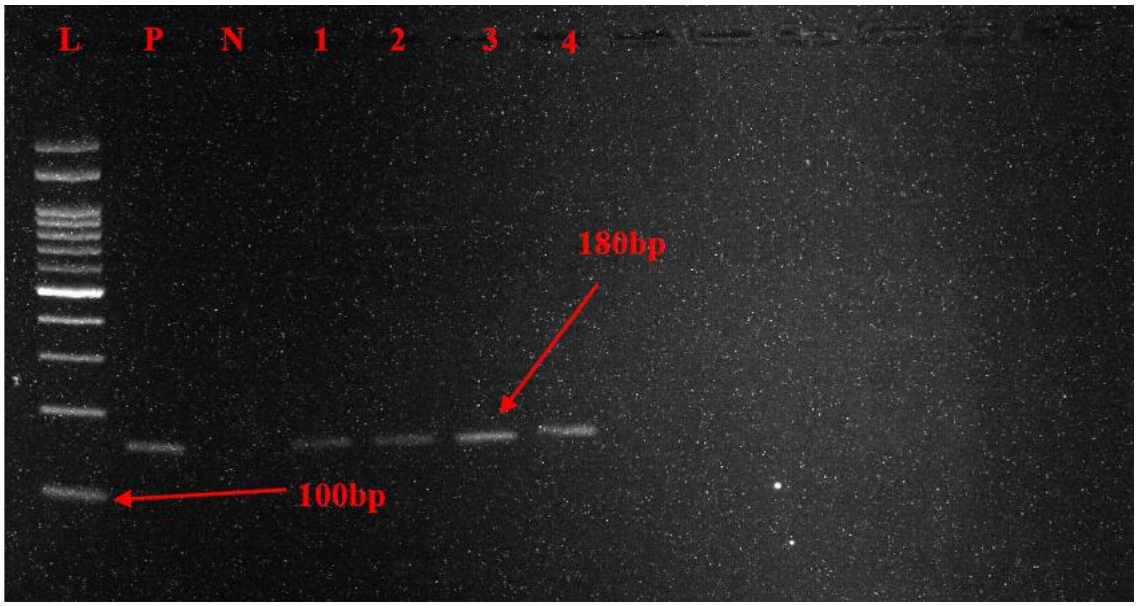
Highest prevalence of *E. coli* was recovered from water samples (52%) followed by intestine (40 %), gills (24%), log swab (24%), skin (16%), muscle (8%), butcher's hand swab (8%) and knife swab (4%). The isolates were characterized morphologically, biochemically and molecular methods.

Results of primer directed amplification of the *stx1* and *stx2* genes are represented in figure 1 and 2 respectively, which show the presence and distribution of the two amplified products when DNA from positive control strain and test isolates were used as template. The size of amplified products was as predicted from the design of the primers that was 180bp for *stx1* primers (Fig. 1) and 255 bp for the *stx2* primers (Fig. 2).

Out of 43 *E. coli* isolates, 4 (9.30%) isolates one each of water sample, butcher's hand swab and two from skin samples were positive for *stx1*, while 7 (16.27%) isolates each one of gills, log swab, two from water samples and three from intestine samples were positive for *stx2* gene and only 1(2.32%) isolate from water sample was positive for both of genes. Similar results for *stx1* and *stx2* genes were reported by Prajapati *et al.*, (2015).

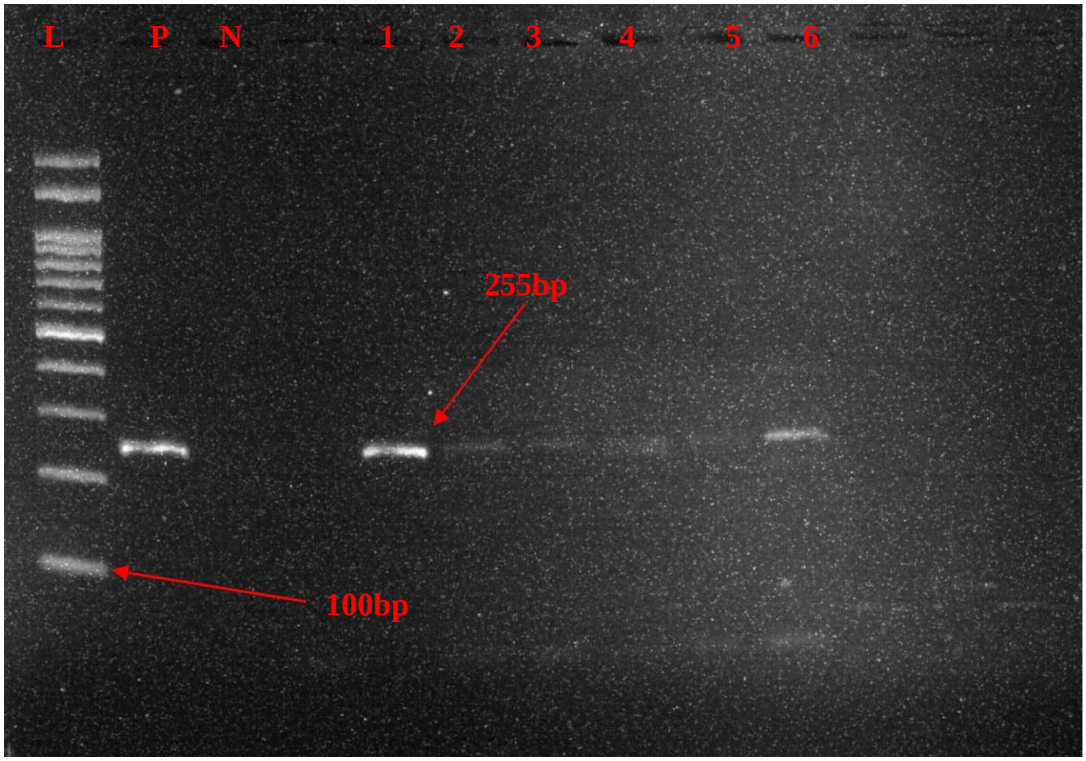
In comparison to present finding high incidence of *stx1* and *stx2* genes were reported by previous worker (Gupta *et al.*, 2013; Shekhar *et al.*, 2017; Bonyadian, 2015). Dhanashree and Mallya (2008); Shekhar *et al.*, (2017) showed a low incidence (0.97%) of *stx1* and *stx 2*. While Kumar *et al.*, (2001) found 3.33 per cent and Alagarsamy *et al.*, (2010) found 4.5 per cent positive for *stx1* and *stx2* genes.

Fig.1 Agarose gel showing PCR amplified product (180bp) for *stx1* gene in *E. coli* isolates



(L- 100bp DNA Ladder, P- Positive control (MTCC, 433), N- Negative control, Lane 1 to 4 positive samples)

Fig.2 Agarose gel showing PCR amplified product (255bp) for *stx2* gene in *E. coli* isolates



(L- 100bp DNA Ladder, P- Positive control (MTCC, 433), N- Negative control, Lane 1 to 4 positive samples)

In present study, all 43 isolates were tested for *in vitro* antimicrobial resistance pattern against 10 commonly used antimicrobial drugs. The antibiogram of all isolates revealed high degree of sensitivity to Chloramphenicol (95.34%) and gentamicin (93.02%) while high frequency of resistance were observed to ampicillin (67.44%) and streptomycin (32.35%). Results revealed that 67.44 per cent of the isolates showed resistance to ampicillin and such a high percent of resistance among the isolates is in agreement with the findings of Ogbonna *et al.,* (2008), Sifuna *et al.,* (2008), Bolarinwa *et al.,* (2011) and Sanyal *et al.,* (2011) but in contrast to this Barbosa (2014) reported all isolates were sensitivity to ampicillin. Higher frequency of resistance to streptomycin (32.35%) was observed during present study. The finding was agreement with previous workers (Adesiyun, 1993; Jouini *et al.,* 2009). However, Ryu *et al.,* (2012) found majority *E. coli* isolated from commercial fish and sea food sensitivity to streptomycin. During the present investigation, 95.34 per cent showed the high frequency of sensitivity to chloramphenicol. The similarly result was observed by Abraham (2011) who isolated *E. coli* from cultured fresh water fish. The present finding is in contrast to previous worker (Ciceron *et al.,* 2008; Bolarinwa *et al.,* 2011) Samuel *et al.,* (2011) who observed the resistance to chloramphenicol. Rocha *et al.,* (2014) isolated *E. coli* from gills, muscle and body surface of farmed fish (Nile tilapias) and observed that high frequency of sensitivity to gentamicin. In present finding similarly result was observed. Looking to the Ciprofloxacin, 76.74 per cent of isolates were sensitive to this antibiotic which is in agreement with the finding of Abraham (2011). However some reports indicated resistance of *E. coli* against Ciprofloxacin (Bolarinwa *et al.,* 2011 and Purushotttam *et al.,* 2011). In present study

60.46 per cent of the isolates were sensitive to ofloxacin but Bolarinwa *et al.,* (2011) recorded 50 per cent resistance against Ofloxacin. This may be due to more frequent use of ofloxacin in the field under their study. Trimethoprim showed sensitivity towards *E. coli* isolates which is related with finding of Teophilo *et al.,* (2002) and Samuel *et al.,* (2011) but in contrast to Jouini *et al.,* (2009) was detected high resistance to Trimethoprim may be due to indiscriminate use of this antimicrobial agent.

Multidrug resistant pathogenic *E. coli* in market fish is considered a sanitary case and may represent a risk to the consumers. However, the presence of non-pathogenic *E. coli* in fish and shellfish should also sound an alert to public health experts, since this bacterium is recognized as an indicator of fecal contamination, possibly indicating the presence of other enteric pathogens.

The most of fresh water fish samples (22.5%) were found to be contaminated or carried *E. coli* infection, which need strict monitoring and surveillance for effective measures for hygiene and sanitary practice. The high percentage resistance of bacterial isolates to ampicillin and streptomycin will be helpful as guidelines for clinical approach in forms of antibiotic therapy. Molecular studies to determine the significance of toxigenic *E. coli* could easily be detected by PCR technique, which is relatively simple, cheap, highly sensitive and specific.

Continuous efforts are required to reduce the antibiotic resistance burden in humans by strict monitoring of antibiotic resistance patterns of *E. coli* from fresh water fish samples and PCR based molecular epidemiological studies for detection of all types of pathogenic as well as zoonotic potential strains of *E. coli* isolates for future research.

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