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Molecular Characterization and Antimicrobial Resistance of *E. coli* Isolated from Fresh Water Market Fish

B.I. Prajapati[1]*, R.N. Gelani[2], A.C. Patel[3], Anjay[4] and C.V. Savalia[5]

[1]Department of Veterinary Public Health and Epidemiology,
Veterinary College, S.D.A.U., Sardarkrushinagar.
[2]Veterinary Officer, Shree Surat Pojarapor, Surat
[3]Department of Veterinary Microbiology, Veterinary College, S.D.A.U., Sardarkrushinagar.
[4]Department of Veterinary Public Health and Epidemiology, Bihar Veterinary College, Patna, Bihar.
[5]Department of Veterinary Public Health and Epidemiology, Vanbandhu College of
Veterinary Science & Animal Husbandry, Navsari Agriculture University, Navsari.

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Escherichia coli **are among the most widely prevalent organisms in the environment and have been isolated from many food animals. In this study a total of 134 market fish samples comprising 25 each of Catla, Rohu, Mrigal, Grass carp and Common Carp, and 9 Silver Carp were screened for the presence of *E. coli*. Among these samples, *E. coli* could be isolated and characterized biochemically as well as by PCR from 25 (18.66%) samples. Virulence genes detection by PCR study revealed the presence of *stx*1 and *stx*2 genes in 12% and 8% of isolates, respectively. Antimicrobial testing revealed the presence of multidrug resistant pathogenic *E. coli*. 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.**

Keywords: Antibiotic Sensitivity Test, Enteropathogenic *E. coli*, Fish, PCR

Escherichia coli are Gram negative, rod-shaped bacteria that inhabit the intestinal tract of humans as well as warm-blooded animals. Although most of the *E. coli* strains are non-pathogenic to human beings, some are known to cause intestinal and extra-intestinal diseases[1,2]. Based on pathogenicity attributes, *E. coli* has been classified into different pathotypes. The pathotypes which produce shiga toxin, a potent cytotoxin, are referred as shiga toxin producing *E. coli* (STEC) or verotoxin producing *E. coli* (VTEC). The major virulence factors present in STEC are shiga toxin 1 encoded by the *stx*1 gene and shiga toxin 2 encoded by *stx*2 gene. STEC has been reported globally as an emerging zoonotic diarrhoeal pathogen that causes severe clinical symptoms in humans *viz.*, Haemorrhagic Colitis (HC) and Haemolytic Uremic Syndrome (HUS) in addition to diarrhoea[3]. Ruminants are considered as the primary reservoir of STEC and human beings may acquire infections primarily through consumption of contaminated food of animal origin. Other sources of infection may include fish, vegetables, fruits, contaminated

* To whom all correspondence should be addressed.
E-mail: dr.biprajapati3143@gmail.com

drinking water, or direct contact with faeces of infected persons. In India, information regarding prevalence of STEC is scarce and has been reported from fish[4], cattle[5], sheep[6], beef[7] and human faeces[7]. In the present study, attempts were made to isolate *E. coli* from market fish samples collected from different fish markets and were characterized by using standard microbiological methods. They were further tested for the presence of 16S rRNA, shiga toxin virulence genes (*stx*1and *stx*2) by PCR and their susceptibility to various antibiotics.

MATERIALS AND METHODS

Sample collection

A total of 134 fresh water fish samples including 25 each of Catla (*Catla catla*), Rohu (*Lebeo rohita*), Mrigal (*Cirrhinus mrigala*), Grass carp (*Ctenophyryngodon idella*) and Common Carp (*Cyprinus carpo*), and 9 silver carp (*Hypophthalmichthys nobilis*) were collected from fish markets of Navsari, Gujarat, India form March-2011 to Febuary-2012. All samples were collected aseptically in sample collection bags and brought to the laboratory over ice.

Isolation and biochemical characterization

Gills and intestine of fish were separated aseptically from the fish samples and about 10 g were inoculated in MacConkey's broth and incubated at 37°C for 18 h. A loupfull of enriched culture was further streaked on MacConkey's agar and incubated at 37°C for 18-24 h. Pink coloured characteristic colonies from MacConkey's agar were sub cultured on Eosin methylene blue (EMB) agar. The sub cultured colonies showing greenish metallic sheen were subjected to a battery of biochemical tests namely Indole production, Methyl red, Voges proskauer, Simon's citrate agar, Nitrate reduction etc. were done for the confirmation of *E. coli* as proposed by Edward and Ewing[8].

Extraction of genomic DNA

The biochemically confirmed isolates were inoculated into 5 ml Luria Bartani (LB) broth and incubated at 37°C for 18 h. After incubation, 1 ml of broth culture was centrifuged at 8000 rpm for 10 min and pellet was resuspended in 200 µl nuclease-free water. DNA extraction was carried out from resuspended pellets using DNeasy Blood and Tissue Kit (Qiagen) following manufacturers protocols.

Detection of *E. coli* using Eubacterial universal primers

A PCR targeting 16S rRNA was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5'CGG YCC AGA CTC CTA CGG3') and reverse (5'TTA CCG CGG CTG CTG GCA3') primers each[9], 3 µl of genomic DNA and nuclease free water upto 25 µl.

The reaction was standardized in a thermal cycler (Applied Biosystem, Veriti™ 96-well Thermal Cycler) with initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 90 s, 60°C for 90 s and 72°C for 120 s. Final extension was carried out at 72°C for 10 min. The amplified product (202 bp) was electrophoresed in 2% agarose gel (Biogene, USA) stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Axygen, USA).

Detection of virulence genes by multiplex PCR

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' CTC GAC TGC AAA GAC GTA TG 3', stx2: 5'ACG ATA GAC TTT TCG ACC CAA CAA 3') and reverse stx1: 5' TCG TTC AAC AAT AAG CCG TA 3', stx2: 5'AAA TAA CTG CCC GGT GGG GT 3') primers[10], 3 µl of genomic DNA and nuclease free water upto 25 µl.

The amplification condition was optimized using the cycle conditions of initial denaturation at 94°C for 120 s, 25 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 60 s and extension at 72°C for 60 s, followed by additional extension for at 72°C for 10 min. Amplified products were separated in 2% agarose gel and stained with ethidium bromide (0.5 µg/ml). Standard molecular size marker (50 bp DNA ladder) was included to measure the product size of 199 bp for *stx*1 and 140 bp for *stx*2. DNA fragments were observed and photographed in gel documentation system (Biorad, USA).

Antimicrobial resistance

The antimicrobial susceptibility test of isolates was studied using agar disc diffusion method described by Bauer et al[11]. The antibiotics discs used to test the isolates were ciprofloxacin (10 µg), amikacin (30 µg), cefixime (5 µg),

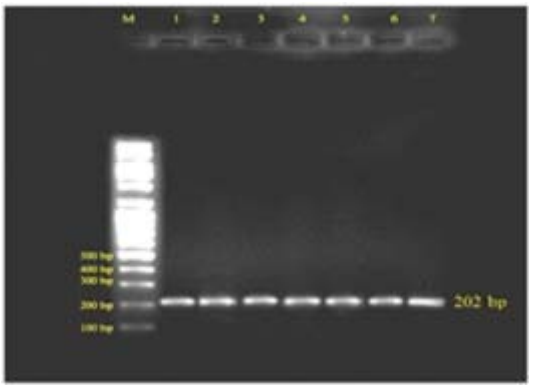
enrofloxacin (10 µg), ofloxacin (5 µg), trimethoprim (5 µg) and oxytetracycline (30 µg). The isolates were propagated on autoclaved Mueller Hinton broths (HiMedia, India) for 18 hrs. at 37°C. Approximately 100 µl of the inoculum was spread on Mueller Hinton agar using sterile spreader and antibiotic discs were placed onto the plate using sterile forceps. The plates were incubated at 37°C for 24 h and observed for zone of inhibition. The results were interpreted as sensitive, intermediate and resistant based on diameter of zone of inhibition produced by different isolates.

RESULTS AND DISCUSSION

The bacteriological examination of market fish samples (n=134) revealed the presence of *E coli* in 25 (18.66%) samples. The isolates were characterized morphologically, biochemically and molecular methods. In PCR study targeting 16S rRNA gene, all isolates were found positive to give specific amplicon of 202 bp size (Fig-1). The detection of *E. coli* in different fish species were found as *C. catla* (28%), *C. carpo* (24%), *H. nobilis* (22.22%), *L. rohita* (16%), *C. mrigala* (12%) and *C. idella* (12%). The presence of approx. 19 per cent *E. coli* in common market fish samples in the present finding was in agreement with earlier studies which reported between 13 and 28% prevalence[12,13,14,15,16,17,18]. However, in contrast to these findings the prevalence of *E. coli* was reported to be 5-9 per cent[19,20,21]. High prevalence of 100% was also reported in fish[22].

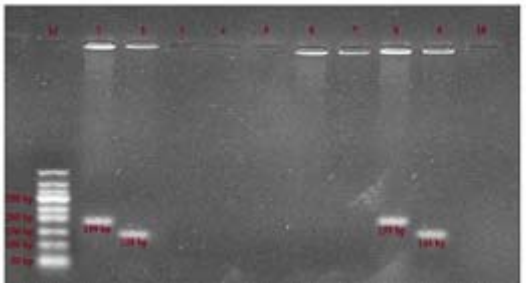
All *E. coli* isolates were subjected to multiplex PCR targeting virulence genes *viz*., *stx*1 and *stx*2 and it was found that 12% (3 of 25) revealed presence of *stx*1 gene and 8% (2 of 25) carried s*tx*2 gene (Fig-2). The presence of *stx*1 and *stx*2 was reported to be 0.97 to 4.5% were reported by other workers[23,24,4]. The presence of *E. coli* as well as verotoxigenic *E. coli* O157:H7 in fish meal was also investigated by some other authors[25,26,27,28].

Antibiotic susceptibility testing of the isolated *E. coli* showed that 72% isolates were sensitive to enrofloxacin, 68% each to ciprofloxacin and ofloxacin, 52% to trimethoprim and 48% to amikacin. However, only 8% showed sensitivity to cefixime but none of the isolates were sensitive to oxytetracycline. Among resistant group, 80% were found resistant to oxytetracycline, 52% to cefixime and 12% to trimethoprim. Intermediate resistance was evidenced against Oxytetracycline (20%), Enrofloxacin (28%), Ciprofloxacin and Ofloxacin (32% each), Trimethoprim (36%), Cefixime (40%) and Amikacin (52%). Looking to the Ciprofloxacin, 68 per cent of isolates were sensitive to this antibiotic which is in agreement with the finding of Abraham[29]. However some recent reports indicated resistance of *E. coli* against Ciprofloxacin[30,17]. In present study 68 per cent of the isolates were sensitive to Ofloxacin but Bolarinwa et al.[30] recorded 50 per cent resistance against Ofloxacin. This may be due to more frequent use of ofloxacin in the field under their study while Hleba et al.[31] recorded 100% sensitivity towards *E. coli*. Trimethoprim showed sensitivity towards *E. coli*



Lane M: 100 bp DNA marker Lane 1-7 : Positive samples

Fig. 1. Agarose gel showing PCR amplified product (202 bp) for 16s rRNA gene in *E. coli* isolates



Lane M: 50 bp DNA marker
Lane 1&8 : Positive for stx 1 gene
Lane 2 & 9 : Positive for stx 2 gene
Lane 3 - 7 : Nagative samples

Fig. 2. Agarose gel showing PCR amplified product 199 bp for for stx 1 and 140 bp for stx 2 genes in *E. coli* isolates

isolates which is related with finding of Teophilo et al.[32] and Samuel et al.[33], in contrast Jouini et al.[34] was detected high resistance to Trimethoprim. None of the isolates recovered from the fish samples showed resistance to Amikacin. This finding is in total agreement with that of Jouini et al.[34], Ryu et al.[35] and Saynal et al.[36]. Results revealed that 80 per cent of the isolates showed resistance to Oxytetracycline and none of the isolates was sensitive to Oxytetracycline, was totally difference finding than Hleba et al.[31] reported 100 % sensisitivity towards Oxytetracycline. 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.

CONCLUSION

The isolation results showed the presence of *Escherichia coli* in fish which is of public health importance. There is need to educate the consumers on the side effects associated with improper handling of fresh fish and consumption of raw or improperly cooked fish. *Escherichia coli* are often used as an indicator for faecal contamination; however because of the ubiquitous nature of this organism in the tropics, this association is questionable. Some strains of *E. coli* are capable of causing food borne disease, ranging from mild enteritis to serious illness and even death. Unhygienic fish handling practices and inadequate cooking may contribute to the spread of pathogen. Appropriate surveillance methods are needed to keep the levels of such food borne infections to a minimum. In view of the fact that *E. coli* isolated during the course of our study may be pathogenic to humans, it is therefore recommended that people associated with selling of fishes should observe good hygiene practices and maintain sanitary conditions. Fish must be properly cooked before it is consumed to avoid food borne infection associated with contaminated and improperly cooked fish.

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