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M Soma Sekhar

Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

N Mohammad

Department of Veterinary Microbiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

T Srinivasa Rao

Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Correspondence

M Soma Sekhar

Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

Serotypes of sorbitol-positive shiga toxigenic *Escherichia coli* (SP-STEC) isolated from freshwater fish

M Soma Sekhar, N Mohammad Sharif and T Srinivasa Rao

Abstract

A total of 104 strains of *Escherichia coli* isolated from the intestinal contents of freshwater fish (*Catla catla*) (n=150) were subjected for virulence characterization by multiplex PCR. One or more virulence genes were detected in a total of 18 (17.3%) isolates viz., shiga-toxin type-1 (*stx1*) in 18 (17.3%) isolates, intimin (*eaeA*) in eight (7.6%) isolates, shiga-toxin type-2 (*stx2*) in four (3.8%) isolates and enterohemolysin (*hlyA*) in four (3.8%) isolates. All the 18 isolates have shown pink colonies on Sorbitol-MacConkey agar (SMAC) indicative of sorbitol fermenting non-O157 serotype. Serotyping was performed to identify sorbitol positive (SP)-shiga toxigenic *E. coli* (STEC) isolates. Diverse serotypes of SP-STEC detected include O120 (three isolates), O141 (two), O63 (two), rough (two) and O126 (one), whereas eight isolates were found to be 'O' group untypable (UT). This was the first report on the detection of SP-STEC serotypes from freshwater fish in Andhra Pradesh, India.

Keywords: *E. coli*, freshwater fish, PCR, SP-STEC, serotyping

1. Introduction

Fish is an important source of digestible proteins in human diet [1]. Fisheries sector in India has made rapid strides in recent years and has been considered as sunrise sector being a major foreign exchange earner in the Indian economy [2]. Andhra Pradesh ranks second in freshwater fish production in India [2]. The aquaculture in India mainly constitute Indian major carps viz., the Catla (*Catla catla*), the rohu (*Labeo rohitha*) and the mrigal (*Cirrhinus mrigala*), which contribute to over 90 percent of the total Indian aquaculture production [3]. Foodborne diseases are a growing public health problem worldwide. Being a highly perishable product, fish and fish products accounted for the 17% of food-borne disease outbreaks in United States [4]. *E. coli* is considered as one of the most important food-borne pathogen in fish and fish products [5]. *E. coli* has been widely applied as a microbiological quality parameter and as an indicator organism of faecal contamination of fish products [5]. Shiga toxigenic *E. coli* (STEC) is known to cause a number of food-borne illnesses in human beings mediated by the production of virulence factors such as shiga-toxin type-1 (encoded by *stx1*), shiga-toxin type-2 (encoded by *stx2*), intimin (encoded by *eaeA*) and enterohemolysin (encoded by *hlyA*) [6]. The STEC family is diverse with more than 200 serotypes identified, of which more than 100 serotypes are of non-O157 type, reported to be responsible for outbreaks of haemorrhagic colitis (HC) or haemolytic uremic syndrome (HUS) in addition to the classical *E. coli* O157:H7 [7]. Sorbitol MacConkey (SMAC) agar has been described as a useful strategy for the differentiation of *E. coli* O157:H7 from non-O157 serotypes [7]. *E. coli* O157:H7 produces colourless colonies on SMAC agar (sorbitol negative, SN), whereas non-O157 serotypes give pink colour colonies due to sorbitol fermentation (sorbitol positive, SP) [7]. There is a need of thorough control over microbiological quality of fish, as bacterial microbiota strongly determines the quality of fresh fish. Considering the public health significance of STEC in fish, the present study was undertaken for the detection of virulence genes (*stx1*, *stx2*, *eaeA* and *hlyA*) in *E. coli* isolates from freshwater fish and identification of common serotypes of STEC from freshwater fish (Indian major carp, *Catla catla*) in Andhra Pradesh, India.

2. Materials and Methods

2.1 Sample collection and processing: Intestinal contents (approximately 10g each) from raw freshwater fish (*Catla catla*) (n=150) were collected from the fish markets of Andhra Pradesh, India.

Homogenized intestinal samples were inoculated into Trypticase soya broth (TSB) and incubated aerobically at 37°C for 24 h. Enriched samples were streaked onto MacConkey agar and eosin methylene blue (EMB) agar, incubated at 37°C for 24 h. Lactose fermenting pink colonies and metallic sheen colonies were picked up onto nutrient agar slants as pure culture and subjected to standard biochemical tests for *E. coli* [8].

2.2 Characterization of virulence genes by multiplex PCR:

E. coli isolates were subjected to multiplex (m)-PCR for the detection of *stx*₁, *stx*₂, *eaeA* and *hlyA* genes as described by Paton and Paton [9]. Oligonucleotide primers used and their

expected amplicon sizes were listed in Table-1. The m-PCR was carried out in 10 µl reaction volume containing 5.0 µl of PCR Master Mix (Takara Bio Inc), 0.24 µl of each forward and reverse primer (10.0 pmol/µl), 2.08 µl of nuclease free water and 1.0 µl of DNA template (50 ng/µl). Samples were subjected to 35 cycles, each consisting of 60 sec of denaturation at 95°C, 120 sec of annealing at 65°C and 90 sec of elongation at 72°C for the first 10 cycles, decrementing annealing temperature to 60°C by cycle 15 (decrementing 1°C in each cycle); 60 sec of denaturation at 95°C, 120 sec of annealing at 60°C and 90 sec of elongation at 72 °C from cycle 15 to 25 and incrementing elongation time from 90 sec to 150 sec from cycles 26 to 35 (incrementing 6 sec in each cycle).

Table 1: Oligonucleotide primers used for detection of virulence genes in *E. coli*

Primer	Primer sequence (5'-3')	Target gene name	Amplicon size	Reference
<i>Stx 1</i>	F: ATAAATCGCCATTTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	Shiga toxin-1	180	Paton and Paton [9]
<i>Stx 2</i>	F: GGCACGTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	Shiga toxin-2	255	
<i>eaeA</i>	F: GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	Intimin	384	
<i>hlyA</i>	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	Hemolysin	534	

2.3 Growth on Sorbitol MacConkey (SMAC) agar: Isolates positive for one or more virulence genes were streaked onto SMAC agar plates, incubated at 37°C for 24 h and observed for pink colour colonies (non-O157:H7) and colour less colonies (*E. coli* O157:H7) [7].

2.4 Serotyping of STEC isolates: Serotyping of STEC isolates on the basis of their 'O' antigen was done at National *Salmonella* and *Escherichia coli* Centre (NSEC), Central Research Institute (CRI), Kasauli, Himachal Pradesh, India.

3. Results and Discussion

Of the 150 samples examined, *E. coli* was isolated from a total of 104 (69.3%) samples. In an earlier study from Punjab (India)

[10], 48.9% incidence of *E. coli* was reported from raw fish. Virulence characterization by m-PCR revealed presence of one or more virulence genes in a total of 18 (17.3%, 18/104) isolates (Fig. 1). All these 18 (17.3%) isolates were positive for *stx1* gene, where as *eaeA* gene was detected in eight (7.6%) isolates, *stx2* gene in four (3.8%) isolates and *hlyA* in four (3.8%) isolates. Incidence of STEC in fish and fish products was reported to be 0.8% in Cochin (Kerala, India) [11] and 5.0% in Mangalore (Karnataka, India) [12] by previous workers. In a study on the virulence characterization of *E. coli*, 66.6 and 44.4% incidence of *stx1* and *stx2* genes, respectively in *E. coli* isolated from raw fish in Punjab was reported [10].

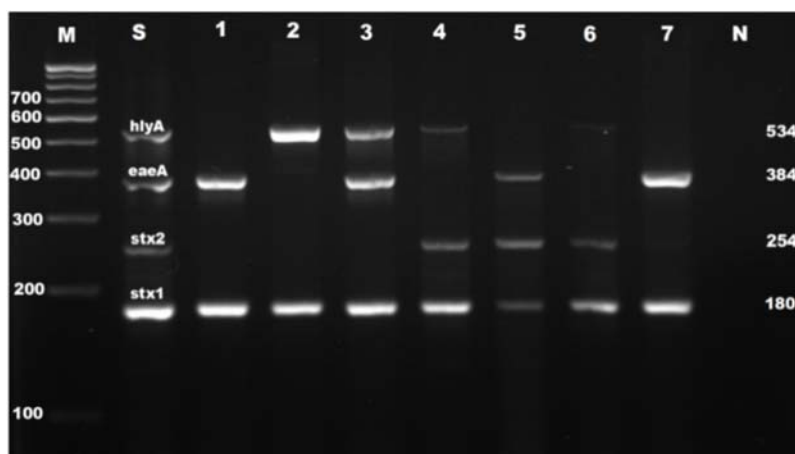


Fig 1: Multiplex PCR amplicons of *Stx 1*, *Stx 2*, *eaeA* and *hlyA* genes. Lane M: Molecular weight marker (100 bp), Lane 1: DNA standard of *E. coli* carrying virulence genes *Stx 1* (180 bp), *Stx 2* (255 bp), *eaeA* (384 bp) and *hlyA* (534 bp), Lane 2-7: *E. coli* isolates from freshwater fish positive for virulence genes, Lane 8: Negative control.

In the present study, about 12 (11.5%) isolates were found to be multi-virulent possessing more than one virulence gene viz., one isolate for *stx1* and *stx2*; one isolate for *stx1* and *hlyA*; six isolates for *stx1* and *eaeA*; one isolate for *stx1*, *stx2* and *eaeA*;

two isolates for *stx1*, *stx2* and *hlyA*; and one isolate for *stx1*, *eaeA* and *hlyA*, whereas six isolates carried *stx1* gene alone. Similar trends of detection of multiple virulence genes was observed in a study by Rao [13]. The presence of shiga toxins

(*stx1* and *stx2*) induces microvascular changes [14]. Intimin (*eaeA*) aids in the adherence to intestinal villi and production of attaching/effacing lesions, while enterohaemolysin (*hlyA*) enhances the effects of shiga toxins [14].

On SMAC agar plates, all the 18 STEC isolates have shown sorbitol fermenting pink colour colonies indicative non-O157 serotype. Serotyping of sorbitol positive (SP)-shiga toxicogenic *E. coli* (STEC) isolates revealed five different serotypes viz., O120 (3 isolates), O141 (two isolates), O63 (2 isolates), rough (2 isolates) and O126 (1 isolate) serotypes, whereas eight isolates were found to be 'O' group untypable (UT). *E. coli* serotypes viz., O5, O11, O17, O28, O41, O58, O69, O103, O168 and O170 were reported from raw fish in a study from Punjab (India) [10]. In another study, O20, O17, O53, O78, O86, O22, O24, O46, O110 and O153 accounted for the predominant serotypes detected in fish STEC isolates [13]. Classical STEC serotypes like O157, O111, O26 were not detected from freshwater fish in the present study. However, O126 serotype of SP-STEC detected in the present study was reported to be pathogenic to humans [15].

4. Conclusion

The present study revealed an overall 17.3% incidence of SP-STEC in freshwater fish of Andhra Pradesh, with majority of strains harboring multiple virulence genes. Detection of pathogenic SP-STEC O126 serotype in the present study represents a risk to the consumers of fish and fish products in the region. Awareness on the consequences of consumption of raw or undercooked fish as well as measures to assess microbiological quality of fish and fish products should be undertaken in order to safeguard the public health.

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