

Incidence and virulence properties of *E. coli* isolated from fresh fish and ready-to-eat fish products

Bhavana Gupta¹, Sandeep Ghatak², J.P.S. Gill³

1. Dept. of Veterinary Public Health, College of veterinary Science & A.H., MHOW, MP - 453446, India

2. Senior Scientist, Division of Animal Health, ICAR Research Complex for the North Eastern Hill Region, Umroi Road, Umiam, Barapani, Meghalaya 793103, India. 3. School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab - 110041, India

Corresponding author: Bhavana Gupta, email: dr.bvgvetgupta@rediffmail.com

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Abstract

Aim: To investigate the incidence and virulence properties of *E. coli* in fresh fish and ready-to-eat fish products from retail markets of the Ludhiana the present study was conducted.

Materials and Methods: Total of 184 samples comprising 96 raw fish and 88 ready- to-eat (RTE) fish products were collected from Ludhiana and other parts of Punjab and were subjected to suitable microbiological methods for *E. coli* isolation. *E. coli* isolates were subjected for haemolytic activity and indicators of plausible cytotoxicity (lecithinase, protease and gelatinase production), congo red dye binding assay. To assess virulence potential isolates were molecularly screened for *stx1* and *stx2* genes.

Results: From raw fish samples 47(48.95%), *E. coli*, were isolated. From RTE fish products 7(12.96%), *E. coli* were isolated. Overall incidence for *E. coli* was 54 (29.34%). *In vitro* virulence characterization of isolates exhibited that all *E. coli* isolates were haemolytic while indicators of plausible cytotoxicity (lecithinase, protease and gelatinase production) were in the range of 16.67% to 35.19% indicated that though the isolates were haemolytic they were perhaps less likely to be cytotoxic. Congo Red binding assay for *E. coli* isolates revealed that majority (88.89%) of the isolates failed to uptake the dye and only few (11.11%) could bind the dye. Results of serotyping revealed a total of 15 different serotypes among the *E. coli* isolates. More variation was observed among isolates from raw fish samples (12 serotypes) while RTE fish products harboured only 5 different serotypes. Molecular characterization of *E. coli* isolates revealed that PCR screening of isolates revealed that total 39 (72.22%) samples out of 54 *E. coli* isolates were positive for *stx1* gene and 28 (51.85%) of isolates were positive for *stx2* gene. Sources wise, 36 (66.66%) of isolates from raw fish and 3(5.55%) of isolates from RTE fish products were positive for *stx1* while and *stx2* gene could be detected in 24(44.44%) isolates from raw fish and 4(7.4%) isolates from RTE fish products. Interestingly, about 20% (37.03%) isolates were positive for both *stx1* and *stx2* genes. Among these multivirulent isolates majority (n=18) belonged to raw fish samples compared to a few (n=2) from RTE fish products.

Conclusion: The results of the present study highlighted the possible risks to consumers of fish and fish products in the region that demand action to address this public health concern.

Key words: *E. coli*, fish, incidence, ready-to-eat fish products, serotyping

Introduction

A heterogeneous group of microorganisms are usually reported to be associated with different body parts of fish. Among the many pathogens associated with fish foods *E. coli* have been identified as important one and is present as normal flora in lower intestine of man as well as animals. *E. coli* is considered major microbial pollutant of water and originates due to contamination of various water bodies with human and animal excreta. Fish and fish products usually acquire contamination from surrounding polluted water or from usages of non-potable water in their preparation and processing. Wide variety of infections are caused by *E. coli* in human including foodborne illnesses that may range from diarrhoeal disease to life threatening haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) due to shiga toxic *E. coli* [1]. Even after the rising importance of fish food in human diet, studies

related to prevalence of common foodborne pathogens in fish food have been relatively less explored in India. Relative paucity of data may be due to public un-awareness and less resources for diagnosis and documentation of the fish foodborne pathogens.

To assess virulence potential of bacterial isolates gelatinase, lecithinase, protease, haemolysin production and congo red dye binding test [2] may be use-full at primary screening level as they may play an important role in disease production by acting as hydrolytic enzymes (gelatinase, lecithinase, protease) or by causing lysis of host cell (haemolysin) or acting as cytotoxic to host cells [3-5]. There is lack of data on the virulence potential of foodborne pathogens from fish particularly molecular characterization data which may aid in development of rapid and efficient detection.

Therefore keeping in view the importance of fish foodborne pathogens the present study was conducted

Table-1. Oligonucleotide primers used for *E. coli*

Gene	Primer sequence	Reference
stx1	F:ATAAATCGCCATTCGTTGACTAC R:AGAACGCCCACTGAGATCATC	Paton and Paton, 1998
stx2	F:GGCACTGTCTGAAACTGCTCC R:TCGCCAGTTATCTGACATTCTG	Paton and Paton, 1998

Table-2. Incidence of *E. coli*, in fresh fish samples and RTE fish products

Sample type	<i>E. coli</i>
Raw fish samples (n=96)	47(48.95%)
RTE fish products (n=88)	7(12.96%)
Total (n=184)	54 (29.34%)

Table-3. Virulence traits for *E. coli* isolates from fish and fish products

Virulence trait(s)	<i>E. coli</i> isolates (n=54) from various source(s)		
	Raw fish (n=47) (%)	RTE fish products (n=7) (%)	Total (n=54) (%)
Haemolysis	47 (100)	7 (100)	54(100)
Lecithinase	7 (14.89)	2 (28.57)	(16.67)
Protease	10(21.27)	1(14.28)	11 (20.37)
Gelatinase	17(36.17)	2 (28.57)	19 (35.19)
Congo red binding test	5(10.63)	1 (14.28)	6 (11.11)
stx1 positive	36(76.59%)	3(42.85%)	39(72.22%)
stx2 positive	24(51.06%)	4(57.14%)	28(51.85%)
Both stx1 and stx2 positive	18(38.29%)	2(28.57%)	20(37.03%)

to study the prevalence of foodborne *E. coli* in fish and fish products and to characterize foodborne *E. coli* isolates prevalent among fish and fish products.

Materials and Methods

A total of 184 samples comprising 96 raw fish and 88 ready-to-eat fish products were collected. Swabs of slime layer, gills and muscle parts of raw fishes weighing approximately 50g and samples of processed ready-to-eat fish products like fried fish, fish kabab were collected from retail roadside food points/ hotels/ restaurants, meat shops of Ludhiana. Approximately 50g of raw fish and/or RTE meat products samples were collected in sterile polythene zipper pack and transported to laboratory on ice and stored at 4°C till further processing. All samples were processed for *E. coli* isolation within 24 hr of arrival in the laboratory. For isolation of *E. coli* MacConkey Lactose Broth (MLB, Hi-media) [6] was used as enrichment broth and Eosin Methylene Blue Agar (EMB, Hi-media) [7] were used as selective and differential media. Presumptive isolates were identified as *E. coli* on the basis of IMViC reaction, H₂S production (TSI test), catalase and sugar fermentation as per standard techniques [4]. To investigate various virulence traits, isolates were subjected to Gelatinase, lecithinase, protease, haemolysin assays and congo red dye binding test [2]. Isolates were streaked onto Tryptose Soya Agar (TSA, Hi-Media) plates with 0.03% Congo Red dye (SRL) and were incubated at 37°C for 24 hr. Colonies with intense orange or brick red colour were noted as positive for dye binding and pale and white colonies were considered negative. Serogrouping of *E. coli* isolates were done at Central Research Institute, Kasauli, Himanchal Pradesh, India.

All the presumed isolates were subjected for polymerase chain reaction (PCR) based assays and screened for presence of *stx1* and *stx2* gene. Primers used for all PCR studies are listed in Table1. Template DNA incorporated in PCR reactions were prepared by two different methods. Usually boil and snap chilling method was adopted. Overnight grown brain heart

infusion (BHI, Hi-Media) broth cultures measuring 1.5 ml were taken into sterilized microcentrifuge tubes and were centrifuged at 10,000g for 5 min. The pellets were washed twice with sterile distilled water and after final washing were re-suspended in 1.0 ml sterile distilled water. Tubes were incubated in boiling water bath for 20 min, followed by immediate chilling on crushed ice for at least 20 min. Finally, tubes were centrifuged at 10000 g for 2 min and 5 µl of clear supernatant layer was used as template DNA in PCR assays. But in certain instances where this method did not yield satisfactory results, genomic DNA was extracted by phenol: chloroform method and was employed for PCR assays as per the method of Budelier and Schorr [8].

PCR for *stx*, and *stx*₂ gene of *E. coli*: To investigate the virulence potential of *E. coli*, isolates were subjected to PCR methodology as described by Paton and Paton [9] with necessary adaptations. In the present study no multiplexing was attempted and PCR was optimized with individual primer pairs targeting individual genes (*stx1* and *stx2*). The reference strain of *Escherichia coli* (MTCC 433) maintained in Department of Veterinary Public Health, College of Veterinary Science, Guru Aangad Dev Veterinary and Animal Sciences University, Ludhiana, were revived and employed for the study. Following initial optimization trials, reaction mixture was standardized in 25 µl volume containing 2.5 µl 10x PCR buffer (100 mM Tris-HCl, pH 8.8, 500mM KCl, 0.8% Non idet P 40), 1.5 mM MgCl₂, 0.2 mM dNTP mix, 12.5 pmol µl of each forward and reverse primer, 1 unit Taq DNA polymerase and required quantity of purified DNA template (if template is from boiling and snap chill method then 5 µl, and if by 2nd method then 1 µl). Reaction mixture was cycled 35 times in a thermocycler (Mastercycler Gradient, Eppendorf, Germany) with preheated lid (105°C) under following PCR cycling conditions. Each cycle consisting of 1 min of denaturation at 95°C, 2 min of annealing at 65°C for first 10 cycles, decrementing 1°C in each cycle by 11th cycle to 15th cycle and from 15th cycle to 35th at 60° C and 1.30 min of elongation at 72°C for first 25 cycle

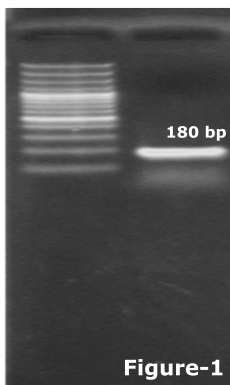


Figure-1

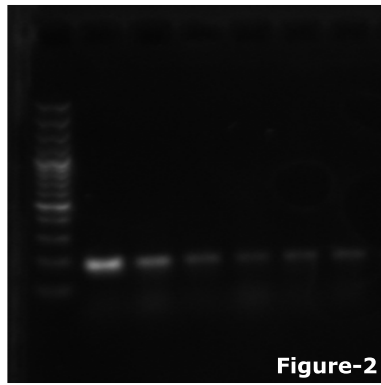


Figure-2

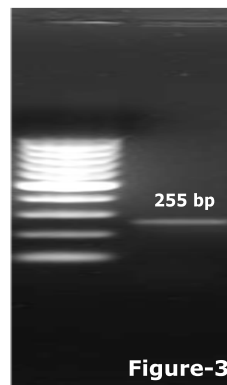


Figure-3

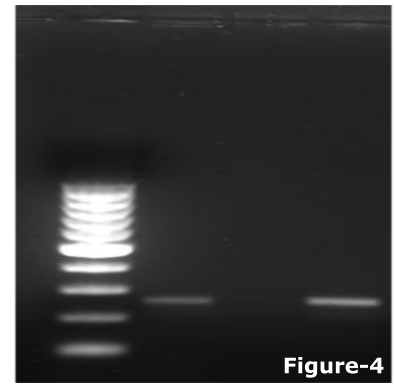


Figure-4

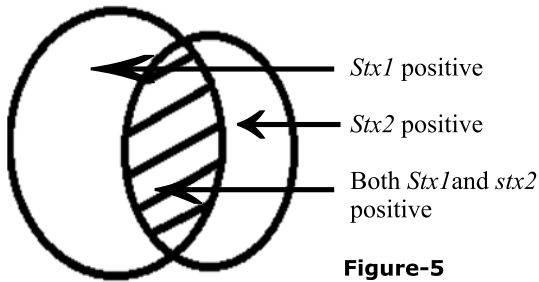


Figure-5

Figure-1. Standardization of PCR for *stx1* gene of *E. coli*; From left to right, Fermentas 100bp plus DNA ladder, reference strain of *E. coli* (MTCC 433)

Figure-2. Standardization of PCR for *stx1* gene of *E. coli*; From left to right, Fermentas 100bp plus DNA ladder, reference strain of *E. coli* (MTCC 433) Positive isolates for *stx1* gene isolate no. 12, 13, 14, 15 and 16.

Figure-3. Standardization of PCR for *stx2* gene of *E. coli*; From left to right, Fermentas 100bp plus DNA ladder, reference strain of *E. coli* (MTCC 433)

Figure-4. Standardization of PCR for *stx2* gene of *E. coli*; From left to right, Fermentas 100bp plus DNA ladder, reference strain of *E. coli* (MTCC 433), Negative control, Positive isolate no. 80 for *stx2* gene.

Figure-5. *Stx1* and *stx2* gene in *E. coli* isolates from fish and fish products

and incrementing 6 seconds in each cycle from 26th cycle to 35th cycle followed by final extension at 72°C for 5 min. Amplified products were analyzed by agarose gel electrophoresis. To ensure quality control of PCR reactions appropriate positive and negative controls were incorporated in each run. On completion of PCR amplified products were analyzed by agarose gel electrophoresis.

Following agarose gel electrophoresis, PCR amplicons were visualized under UV trans-illumination in a Gel Documentation system (G Box, SYNGENE) and data were recorded photographically.

Results and Discussion

On completion of PCR reaction amplified products were analyzed by agarose gel electrophoresis which yielded amplified product of 180 bp (Figure-1) for *stx1* and 255bp (Figure-2) for *stx2*.

Overall incidence for all type of samples was 29.34%. From 96 raw fish samples, 47(48.95%) *E. coli*, were isolated where as from 88 ready- to-eat fish product samples 7(12.96%) *E. coli*, were isolated. This result is in conformity with previous reports from other workers.

Kumar *et al* [10] determined the prevalence of *E. coli* in tropical seafood and documented a prevalence of 47% for faecal coliforms including *E. coli*. *E. coli* was isolated from fishes grown in sewage-fed farms and also from retail market fishes of Kolkata that were contaminated with faecal matter of animal and human origin [11]. Results of serotyping revealed a total of 15 different serotypes among the *E. coli* isolates. More variation was observed among isolates from RTE fish products (only 5 different serotypes) and less in case of raw fish samples (12 serotypes). Serotype O69 (n=26) was found to be associated with maximum number of

isolates. Similar, variations in serotype distribution were also reported previously by Rao [12] who study Shigatoxic *E. coli* in the region. O44 and UT serotypes were common to both type of fish foods (raw and RTE) this may be due to cross over contamination of RTE fish foods from raw fishes. On the other hand serotypes that were exclusive according to their sources comprised of for raw fish samples O11, O17, O28, O41, O58, O69, O103, O168, O170 and Rough and for RTE fish products O5, O100, O172. Some of Serotypes like O5 and O103 were found to be associated among various non -O157:H7 type human diseases[13], were also isolated from samples of fish and RTE fish products of area this indicates that the fish and RTE fish products are harboring potential pathogenic *E.coli*. In the present study no correlation was found between serotypes and *stx* production though it was reported by various worker like [14] found five serotypes viz. O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 positively correlated with *stx* production ability.

Presumptive isolates were identified as *E. coli* on the basis of IMViC reaction, H₂S production and sugar fermentation as per standard techniques. Results were on the expected line with no variations observed. Virulence potentials were investigated *in-vitro* for gelatinase activity on 5% gelatin agar, lecithinase production on egg yolk agar, protease production on 5% skim milk agar, haemolysis on 5% sheep blood agar and Congo red binding assay on tryptic soy agar, containing 30 µg Congo Red dye. Results (Table-3) indicated that all *E. coli* isolates were haemolytic while indicators of plausible cytotoxicity (lecithinase, protease and gelatinase production) were in the range of 16.67% to 35.19%. Results exhibited that though the isolates were haemolytic they were perhaps less likely to be cytotoxic. On the other hand Congo Red binding

assay for *E. coli* isolates revealed that majority (88.89%) of the isolates failed to uptake the dye and only few (11.11%) could bind the dye (Table-3). While this result apparently indicate that majority of the isolates were non-pathogenic, it must be considered that the sensitivity of the Congo red binding assay was only 58.89% [15]. This lack of sensitivity, notwithstanding, many researchers employed this widely popular test as a marker for virulence potential among the organisms [16].

To assess the virulence potential of the *E. coli* isolates PCR protocols were optimized targeting two well known virulence genes-*stx1* and *stx2* as described previously [13] with suitable modifications. PCR screening of isolates revealed that total 39 (72.22%) samples out of 54 *E. coli* isolates were positive for *stx1* gene and 28 (51.85%) of isolates were positive for *stx2* gene. Sources wise, 36 (66.66%) of isolates from raw fish and 3(5.55%) of isolates from RTE fish products were positive for *stx1* while and *stx2* gene could be detected in 24(44.44%) isolates from raw fish and 4(7.4%) isolates from RTE fish products (Table-3). STEC (shiga toxinogenic *E. coli*) may belong to a very broad range of O serogroup, with in the human disease associated strains though those which are *stx2* positive appears to produce serious complications like HUS (haemolytic-uremic syndrome) [13].

Interestingly, about 20% (37.03%) isolates were positive for both *stx1* and *stx2* genes. Among these multi-virulent isolates majority (n=18) belonged to raw fish samples compared to a few (n=2) from RTE fish products.

Similar trend in regard to *stx1* and *stx2* genes was observed [12] However, [12] detected *stx1* in 100% samples, while the findings of the present study revealed *stx1* gene presence in about 67% of the isolates. This difference may be due to the variation in the sampling scheme among these studies. Among a list of virulence factors of *E. coli* shiga toxin 1, shiga toxin 2, intimin, enterohaemolysin are considered important [3]. Of these four factors Shiga toxin 1 & 2 have emerged as most relevant because they play an important role in pathogenesis. Shiga toxins leads to the pathogenesis of haemorrhagic colitis and haemolytic-uremic syndrome by inducing microvascular changes in vivo and are cytotoxic to selected cell lines in vitro. Intimin may helps in pathogenesis by adherence to intestinal villi producing attaching and effacing lesions, and enterohaemolysin may enhance the effects of Shiga toxins [3].

Kumar *et al* [17] conducted a study to screen *Escherichia coli* isolated from various sea foods such as fresh fish, clams and water for the presence of *stx*, *hlyA* and *rfbO157* genes by PCR and reported that 5% of clams and 3% of fresh fish samples were positive for non-O157 STEC. Surendraraj *et al* [18] screened fish and shrimp samples from different retail fish markets in Cochin for their shigatoxic potential; the authors reported significant incidence of shigatoxic *E. coli* in those samples.

Conclusions

This study revealed an overall 29.34% incidence of *E. coli* in fish and fish products which clearly indicate that in the study area fish and fish products do harbour a number of foodborne pathogens. Isolates of pathogens expressed multiple virulence traits indicating that prevalent pathogens were capable of inducing disease in consumers. Of the 54 *E. coli* isolates, 72.22% possessed *stx1* gene and 51.85% isolates carried *stx2* gene with raw fish samples being the major source for virulence genes. Finally the results of the present study indicated possible risks to consumers of fish and fish products in the region that demand action to address this public health concern.

Author's contribution

SG and BG conceptualized and designed the study. BG has performed research experiments. SG, BG and JPSG drafted, revised and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interest.

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