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Research Article

Virulence Gene Profile of *Escherichia Coli* Isolated from Raw Milk and Market Meat in Guwahati City of Assam

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Abstract

Escherichia coli is a commensal organism of the human gut. It is found relatively in low numbers as compared to other commensal bacteria; however, it is the most common cause of food-borne illnesses. The present study aimed to determine the presence of virulence genes *stx1*, *stx2* and *eae* among *E. coli* isolates recovered from unpasteurized milk and raw meat samples. A total of 79 samples including 47 milk and 32 meat samples were collected from different areas of the Guwahati city. All samples were processed for microbiological analysis within 4 hours of collection and the suspected *E. coli* isolates were confirmed by staining and biochemical tests (IMViC and Urease). Virulence genes, viz. *stx1, stx2* and *eae* were detected in the isolates by polymerase chain reaction (PCR) using specific primers. Out of 47 milk and 32 meat samples, 16 (34.04%) and 23 (71.8%) (n=23) revealed the presence of *E. coli,* respectively. None of the isolates from milk was found to be positive for any of the virulence genes while 11 (47.83%) of 23 isolates in meat, were positive for stx2, 2 (8.70%) for stx1 and 1 (4.35%) for eaeA gene. The result of the study suggested that raw and unpasteurized dairy and meat has contaminated with virulent *E. coli* may be associated with potential public health significance. Hence, it is important to take precautionary measures at different levels of the food chain both by the producers and the processors to ensure supply of hygienic and safe dairy and meat products to the consumers.

Keywords: *E. coli*; PCR; Antibiotic; Gel Electrophoresis; Veterinary; Verotoxicity

Abbreviations

E.coli: Escherichia coli; *S. aureus: Staphylococcus aureus; L. monocytogens*: *Listeria monocytogens*; GI: Gastrointestinal; EPEC: Enteropathogenic *E. coli;* EHEC: Enterohemmorhagic *E.coli;* ETEC: Enterotoxigenic *E. coli;* EAEC: Enteroaggregative *E. coli;* EIEC; Enteroinvasive *E. coli;* UPEC; Uropathogenic *E. coli;* TSB: Tryptic Soy Broth; CT-SMAC: Cefixime-Tellurite Sorbitol-MacConkey Agar; HUS: Hemolytic Uremic Syndrome; EtBr: Ethidium Bromide; PCR: Polymerase Chain reaction; FDA: Food and Drug Administration; MLA: MacConkey Lactose Agar; EMB: Eosin Methylene Blue; AST: Antibiotic Susceptibility Test.

Introduction

Escherichia coli is a commensal microorganism, that maintains a symbiotic relationship with its human or animal host with mutual benefits to each other; however, these commensal strains can cause intestinal and extra-intestinal infections, depending on the immunity of the host. The characteristics of the species which support the symbiotic relationship with humans or animals are not fully characterized, although there are many instances which support this fact. One of the reasons is its capability to utilize gluconate in the colon more efficiently than other specific resident species [1]. The pathogenic strains of *E. coli* work by disrupting the normal functions of the host as well as adapting to the new niches.

The pathogenic strains have taken up elements during the course of evolution to cause a broad spectrum of diseases. The different strains can mix with each other and propagate new strains and only the successful combinations with potential virulence factors can persist and be further classified as different pathotypes of *E. coli* [3]. Most infections caused by *E. coli* are acquired via faecal-oral route. The pathogenesis of *E. coli* is a multi-step process consisting of: colonization of mucosal site, evasion of the host defences, multiplication and clinical manifestations. The virulence genes in *E. coli* are found in large segments of DNA, called pathogenicity islands. They may be acquired by horizontal gene transfer. The presence of pathogenicity islands significantly enhances the virulence than their non-pathogenic counterparts [2].

Enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) were first recognized as a causative agent of human disease after an outbreak of haemorrhagic colitis (HC) in the USA in the early 1980's. The main reservoir of EHEC is the bovine intestinal tract. It is the cause of severe clinical syndromes in humans like bloody diarrhea (HC), non-bloody diarrhea and haemolytic uremic syndrome (HUS) [1]. The principal virulence factor of EHEC is its capacity to produce Shiga-toxins, which is also known as Verotoxin. This toxin is encoded by the gene stx, and its name has been derived from Shigella dysenteriae serotype 1, which is cytotoxic for vero cells. Shiga toxins are of two kinds: Shiga-toxin 1 (Stx1) and Shiga–toxin 2 (Stx2). Shiga-toxin 1 is almost identical to the Shiga toxin produced by *Shigella dysenteriae* in respect of its amino acid sequence. Further, Stx1 cannot be serologically distinguished from it, whereas Stx2 has more differences with Shigella toxin and cannot be neutralized by antibodies to Stx1 or Shiga toxin from Shigella dysentriae [4]. Another potential virulence factor, which is associated with EHEC and Enteropathogenic E. coli (EPEC) infections, is attaching and effacing (A/E) factor, which helps in the intimate attachment of the bacteria to the intestinal epithelial cells. They can cause striking cytoskeletal changes, characterized by the accumulation of polymerized actin directly beneath the adhered bacteria, effacement of the intestinal microvilli and frequent formation of pedestal-like structures from the epithelial cells, on which the bacteria can perch [1,5]. Considering the increasing occurrence of resistant strains as well as increasing number of outbreaks due to *E. coli* and other infectious microorganisms, it is necessary to increase food safety for consumer products. Hence rapid, sensitive and specific, should be adopted at different levels of production and processing to ensure safety of milk and meat products. Raw meat may harbour various pathogenic microorganisms which can get infected in aseptic conditions in the slaughterhouses as well as during transportation viz. *Salmonella spp., Campylobacter jejuni/coli, E. coli, S. aureus* and to some extent *L. monocytogens* as well [6,7]. In the North Eastern Region of India, meat is almost a regular dietary item in the daily meals of the ethnic tribes. It is highly nutritive as well as it contains both micro and macro nutrients and makes an important part of the diet [8]. However, very few systematic studies have been conducted so far to ascertain the microbiological quality of milk and meat available in the market in this region. The present study attempted to assess the level of contamination of raw milk and meat samples collected from the market of Guwahati city with verocytotoxigenic *E. coli.*

Materials and Methods

Isolation and Identification of *E. coli*

A total of 30 raw meat and 47 raw bovine milk samples were collected from different parts of Guwahati City during February – March, 2016. The Meat samples included chicken (23), mutton (5) and duck meat (2). All the samples were collected early morning in sterile vials and were properly labelled with an unique identification number, location and date. The samples were transported to the laboratory and processed for bacteriological analysis within 4 hours of collection. The samples were streaked on MacConkey Lactose Agar (MLA) for primary isolation of *E. coli* and incubated aerobically for 24 hours at 37°C. Lactose fermenting colonies of suggestive *E. coli* were further sub-cultivated on Eosin Methylene Blue (EMB) agar for purification and differentiation of *E. coli* from other lactose fermenters. Colonies showing characteristic metallic sheen were further confirmed by staining as well aas biochemical characterisation by Indole, Methyl-red, Voges-Proskaeur, Citrate utilization and Urea hydrolysis tests.

Extraction of DNA

Genomic DNA was extracted from the confirmed E. coli isolates by hot-cold lysis method. The isolates were inoculated in LB broth and aerobically incubated at 37°C for 24 hours. Broth cultures were centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and 1.2 ml of cultured LB broth was again added and re-centrifuged for 5 min at 3000 rpm. The supernatant was discarded and 50 µl of 1X Tris EDTA buffer was added and heated in a boiling water bath at 100°C for 10 min, followed by snap chilling in ice for 10 min and finally centrifuged at 10000 rpm for 10 min. The supernatant was collected in a new tube, while the pellet was discarded. This was directly used as template DNA for PCR for detection of virulence genes.

Polymerase chain reaction

Genomic DNA extracts from *E. coli* isolates were subjected to PCR for screening the presence of *stx1, stx2* and *eae* genes using specific primers reported by previous workers (Table 1). The PCR components were mixed together in 0.2 ml PCR tube to make a final reaction volume of 25 µl, comprising of 12.5 µl of 2X Dream Taq PCR Mastermix (Thermo Scientific), 0.5 µl (10 pmol/µl) of

each primer and 1 µl of DNA template. The reaction was carried out with cycling conditions: 94°C for 30 sec, 30 cycles of 94°C for 30 sec, 47-60°C for 30 sec, 72°C for 45 sec followed by 72°C for 5 min in an Eppendorf Gradient Mastercycler.

Gene	Primers	Sequence	Product size (bp)	Reference
Stx1	Forward	CAGTTAATGTG-GTGGCGAAGG	384	Cebula., *et al.* [9]
	Reverse	CACCAGACAATG-TAACCGCTG		
Stx2	Forward	ATCCTATTCCC-GGGAGTTTACG	482	Cebula., *et al.* [9]
	Reverse	GCGTCATCGTATA-CACAGGAGC		
Eae	Forward	CCCGAATTCGGCA-CAAGCATAAGC	881	Oswald., *et al.* [10]
	Reverse	CCCGGATCCGTCT-GCCCAGTATTCG		

Table 1: Primer used for amplification of virulence genes of E.coli isolates by PCR

The PCR amplified products were separated by 1.5% agarose gel electrophoresis containing ethidium bromide (10 mg/ml) and 1X TAE along with 100 bp ladder. Electrophoresis was carried out at 80V for 60 min. Each PCR product was observed as a single band of expected product size and the results were documented using a gel documentation system (Bio-Rad, USA).

Results and Discussion

A total of 30 meat samples were collected consisting of chicken (23), goat (5) and duck (2) meat. Out of the total 32 samples, 23 (71.85%) were positive for *E. coli*. The graph below shows the number of positive isolates of *E. coli* from each category of meat sample.

Table 2 and Table 3 summarize the results of the present study in respect of detection of virulence genes among the *E. coli* isolates. In all, 34.04% of milk samples and 71.85% of meat samples yielded *E. coli.* The results obtained in this study is in accordance with that of Virpari et al., who obtained 32% milk and milk products to be positive for *E. coli* and Dhanashree et al. who reported isolation of E. coli from 77.8% of meat samples [11,21]. Al-Zogibi *et al*. and Dehkordi et al. reported a lesser percentage of isolates from milk and milk products, 15.93% and 8.33% respectively to be positive for *E. coli* [12,13]. Many other researchers also reported a lower percentage of isolation from meat, viz. 4.6% by Pierrard., *et al*. 15.2 % by Lunna-Herrara., *et al,* 29.02% by Momtaz., *et al.* and 2.8% by Jacob., *et al.* contrary to the present study [16-18,20,21].

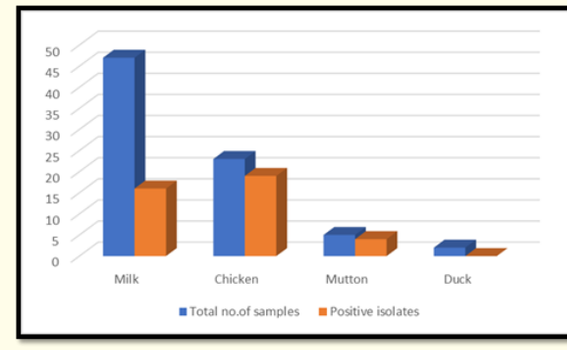

Figure 1: Variation in isolation rates of E.coli among milk and meat samples

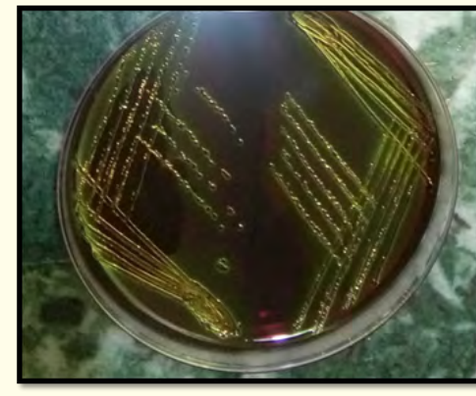

Figure 2: Green Metallic Sheen representing the growth of *E.coli* on EMB Agar.

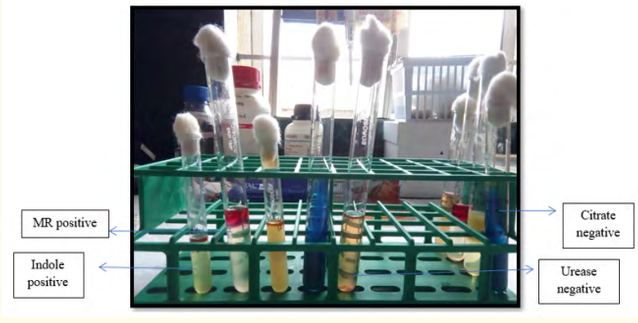

Figure 3: Result of Biochemical Tests.

Table 2: Isolation of *E. coli* from different samples

Category	Total number of samples	Positive *E. coli* isolates
Milk	47	16(34.04%)
Chicken	23	19(82.61%)
Mutton	5	4(80%)
Duck Meat	2	0

Table 3: Depicts the number of *E. coli* isolates with virulence genes for verocytotoxigenicity among the milk and meat samples.

Source	Number of samples	Number of *E. coli* isolates	Number of isolates positive for virulence genes		
			stx1	stx2	eae
Milk	47	16 (34.04)	-	-	-
Chicken	32	23 (71.85)	11	2	1

Figures in parenthesis indicates percentages

Results for Antimicrobial susceptibility test

Antimicrobial Susceptibility Test was carried out by normal Kirby Bauer's disc diffusion method (1966) using Mueller-Hinton Agar. The results obtained have been precisely referred and tallied with previous literature.

Out of 23 isolates, 9 (39.13%) isolates were completely resistant against all the used antibiotics, while 11 (47.82%) isolates were sensitive to at least one isolate and 3 (13.0%) isolates were sensitive to two antibiotics. None of the isolates were sensitive to more than 2 antibiotics which also indicate the rising concern on the antimicrobial drug resistance in the present.

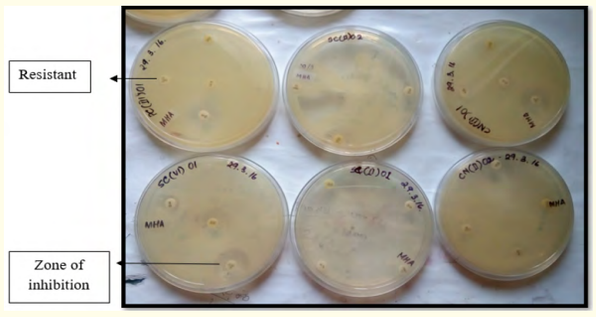

Figure 4: Antibiotic Sensitivity Tests by Disc Diffusion Method.

Table 4: Antibiogram of *E.coli* (n=23) isolates from milk and meat samples

Antibiotics	No. of sensitive isolates	No. of resistant isolates
Penicillin	0	23(100)
Ampicillin	8 (34.78)	15(65.22)
Tetracycline	2 (8.7)	21(91.3)
Streptomycin	6(26.09)	17(73.91)

Figures in parenthesis indicates percentages

Table 5: Detection of virulence genes associated with verotoxicity among *E.coli* isolates from raw milk and meat samples

Virulence genes	No. of isolates with virulence genes (%)
stx1	2 (8.69)
stx2	11 (47.82)
eaeA	1 (4.34)

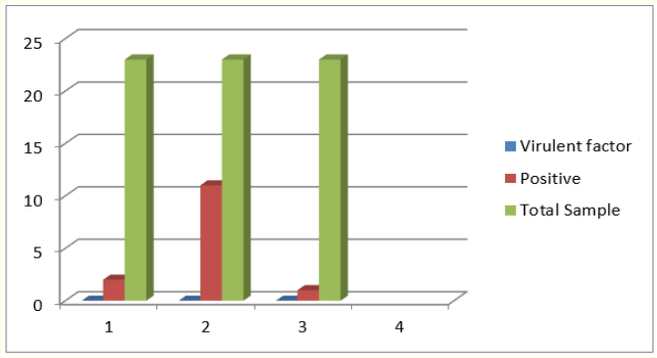

Figure 5: Bar Graph depicts the occurence of virulent genes in *E. coli* isolates.

PCR using specific primers revealed that none of the 16 isolates from milk harboured any of the genes under study. This is in accordance with the findings of Neher., *et al*. who reported the absence of *stx2* gene in *E. coli* isolated from milk samples in and around Guwahati [14]. Mohammadi et al. also reported the absence of stx1 and *stx2* genes while eaeA gene was reported in 8.25% of the isolates from raw milk samples in Iran [15]. The present study indicated prevalence of stx2 in 47.3% of meat samples, followed by stx1 in 8.8% of the samples followed by eaeA in 4.4% of samples. Similar findings were reported by Momtaz *et al.* (2012) who found 36% E. coli strains to be positive for stx1 and stx 2 [17]. Jacob *et al.*, could not find any E. coli isolates from similar sources with stx2 and eae genes while Jakee et al. (2012) reported that *stx2* was the most prevalent (38.46% of isolates positive) among all three genes [19,20]. A lower percentage of detection of these virulent genes was also reported by Pierrard., *et al*. [18].

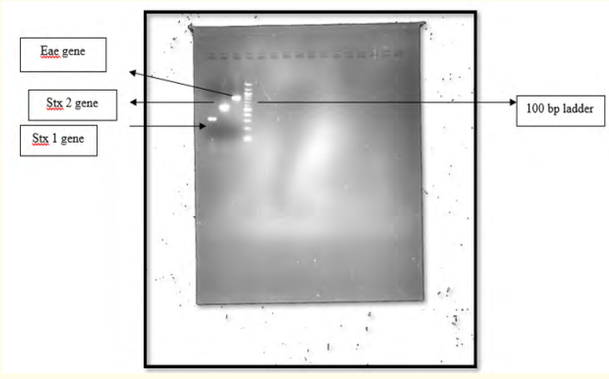

Figure 6: Agarose Gel Electrophoresis showing positive controls for *stx1*, *stx2* and *eae* A genes.

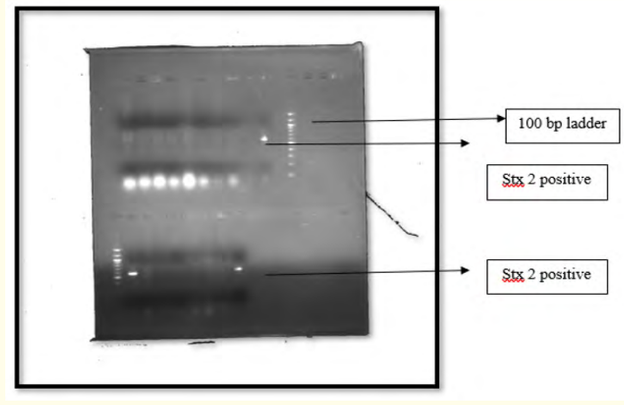

Figure 9: Agarose gel electrophoresis showing positive bands for *stx2* gene.

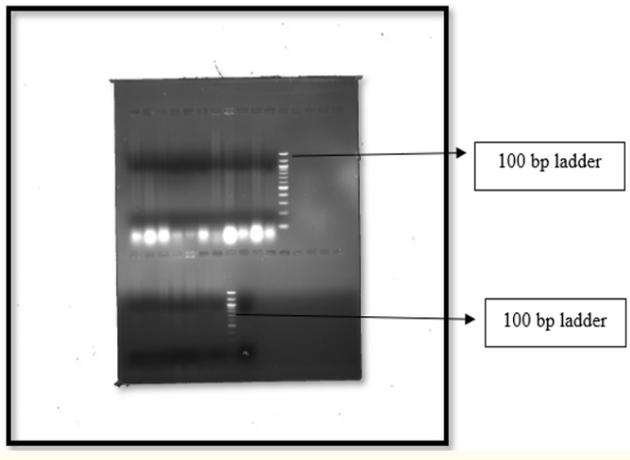

Figure 7: Negative result for agarose gel electrophoreis of *eaeA* genes in samples.

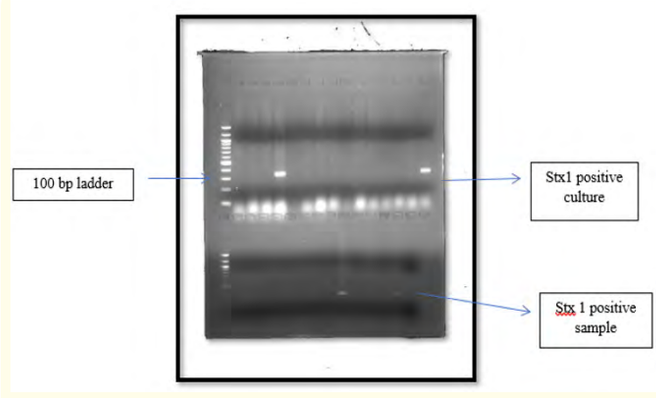

Figure 10: Agarose Gel Electrophoresis showing *stx1* positive bands.

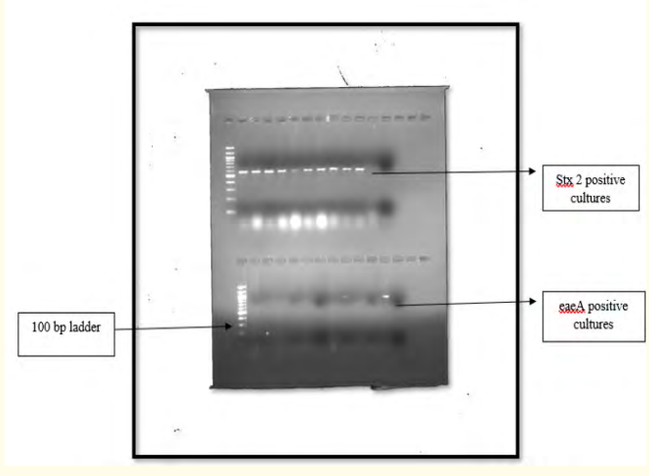

Figure 8: Agarose Gel electrophoresis of meat samples showing positive bands for *stx2* and *eaeA* genes.

Conclusion

Various studies in different regions showed a wide variation in percentage of isolates harboring different virulence genes. The variations can be attributed to factors like geographical area, season, farm size, number of animals on the farm, hygiene status, farm practices, variation in sampling, variation in types of samples, and differences in detection methods adopted. With the adoption of scientific methods of dairy farming and major policy reforms of the government, most of the dairy farms in and around Guwahati are becoming more organized and the farmers are becoming aware of the possibility of transmission of diseases through contaminated milk. However, the results of the present study suggested the need of taking appropriate precautions at different levels of the food chain both by the producers and the processors to ensure supply of hygienic and safe dairy and meat products to the consumers.

Conflict of Interest

The authors declare that there is no conflict of interest.

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