Detection of virulence properties in *E. coli* isolated from packed food products from North East India

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The aims of the current study were to detect the virulence factors and antibiotic resistance of Shiga toxin-producing *E. coli* in packed foods for human consumption from North East (NE) India as one of the major growth sectors in recent decades. After *E. coli* identification with culture method, PCR assay was developed for detection of pathogenic genes (*stx1* and *stx2*). Biofilm formation assay of toxin positive isolates was performed, and antibiotic resistance genes of *E. coli* isolates were determined. The overall incidence of *E. coli* for packed foods from NE India was observed to be 22.22% for cooked, 33.33% for frozen semi-cooked, 54.16% for frozen uncooked, and 45.45% for uncooked. All the *E. coli* isolates exhibited 100% and 87.50% resistance towards Ampicillin and Tetracycline with majority of the isolates being sensitive towards Ciprofloxacin (100%), Ceftazidime (100%), Ofloxacin (100%), Nalidixic acid (90.6%), and Chloramphenicol (87.5%). The amplified products of 180 bp for *stx1* and 255 bp for *stx2* were obtained. Both sorbitol negative and positive isolates were tested for the presence of toxin genes and, of the total 32 *E. coli* isolates, 13 isolates (40.6%) revealed the presence of Shiga toxin genes, of which 6 isolates harbored *stx1* gene; 2 isolates *stx2,* and 5 isolates exhibited the presence of both *stx1* and *stx2* genes. Majority of the isolates harbored *stx1* gene as compared to *stx2* gene. Biofilm formation of the 13 (40.6%) isolates revealed pellicle and distinct film formation at the air-liquid interface. Our results recommended the use of PCR for detection of pathogenic genes of bacteria as a safe, rapid, and accurate method in laboratories and also ready-to-eat foods as well as ready to cook food at all times be handled and stored in clean areas where control ensures the environment is free from *E. coli* contamination.

Keywords: *stx1*; *stx2*; packed foods; NE India.

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Introduction

Packed foods have been described as the status of foods being ready for immediate consumption at the point of sale [1, 2] and such foods in today's time have gained popularity due to smaller household sizes, ageing population, increased mobility, large numbers of itinerary workers, small families or home centered activities, higher incomes, and rising numbers of households where all adults work and less time available for food preparation [3, 4]. But this has also finally created awareness in the society related to increase in food borne diseases resulting from ingestion of bacteria, toxins, and cells produced by microorganisms present in such packed foods as good manufacturing practices and sanitary measures in regard to proper food handling by the food vendors are rarely practiced [5, 6].

Shiga toxigenic *Escherichia coli* (STEC) has been identified as an important cause of gastrointestinal disease in humans where

production of Shiga toxin (*stx*), which inhibits the protein synthesis of the host cells leading to cell death [7]. The morbidity and mortality associated with several recent large outbreaks of STEC diseases have highlighted the threat these organisms pose to public health [8]. Further, attachment of STEC to surfaces and the formation of biofilms may enhance its persistence in a food processing environment and present a risk of contaminating products [9, 10]. Formation of biofilms (STEC) on stainless steel, glass, and polystyrene have been reported by many co-workers and this may be regarded as a survival strategy of bacteria such as *E. coli* O157: H7 [11, 12]. Thus, keeping in view of the above, this study will prove valuable presence of toxin genes (*stx1* and *stx2*) and their virulence properties associated in packed foods from NE India.

Materials and Methods

A total of 83 packaged food samples (different types of meat and vegetables) were procured from various supermarkets of Silchar town, which were either in frozen, ready to eat and cook stage, semi- cooked; cooked, and uncooked form. The samples included different types of parathas, sausages, chicken items, vegetable items, sweet corn, and fruit juices. Collection of samples was done in sterile vials with proper labelling and each vial was assigned a unique identification number. The samples were then transported immediately to the laboratory and stored at 4°C and processed for *E. coli* isolation within 24 hours (h) of collection.

Sample analysis

Sample analyses were performed in two different parts as follows:

(1) For solid food sample: 1g of each food sample was weighed out and homogenized into 9 ml of sterile lactose broth as enrichment media and incubated at 37°C for 24 - 48 h.

(2) For liquid food sample: 1ml of each food sample was homogenized into 9 ml of sterile lactose broth as enrichment media and incubated at 37°C for 24 - 48 h.

Ten-fold dilution of the homogenates were made and 0.1 mL of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the homogenate were plated in MacConkey agar using pour plate method followed by incubation of the plates at 37°C for 24-48 h. Sub-culturing of the isolates was performed on EMB (Eosin Methylene Blue) agar plates followed by incubation at 37°C for 18-24 hours. Strains were stocked on nutrient agar stabs and stored at 4°C for further studies.

Morphological and biochemical identification

All strains belonged to environmental isolates in our collection and they were further characterized based on morphological, cultural, and biochemical characteristics.

The isolates were stained with Gram's staining method and observations were particularly made on shape, size, color, odor, arrangements, and motility. Cultural characteristics were observed on Eosin Methylene Blue (EMB) Agar plates, MacConkey agar plates, and nutrient agar slants. On solid medium the colonies were examined for shape, size, surface, elevation, edge, color, consistency, opacity, and emulsifiability. Color of the isolates varied with specificity of bacteria to the media used. Finally, a series of IMViC tests along with urease and triple sugar iron tests were employed to categorize the bacteria in its respective genera.

In vitro antibiotic sensitivity test

Antimicrobial susceptibility test was performed on Mueller-Hinton agar (MHA) using Kirby-Bauer disc diffusion method [13]. A 24-hour young culture plate was used for inoculation into BHI broth which was then incubated for 18-24 hours at 37°C. About 100-200 µL of the inoculum was poured on MHA plates and spread uniformly. Each antibiotic disc was gently placed and pressed down with a pair of sterile forceps on the medium surface for adhesion and the plates were then kept under incubation aerobically in inverted position at 37°C for 18-24 hours.

Antibiotic discs (Hi-Media, Mumbai, India) used for the present study are Chloramphenicol 30 µg, Ampicillin 10 µg, Ciprofloxacin 5 µg, Ceftazidime 30 µg, Tetracycline 10 µg, Ofloxacin 5 µg, and Nalidixic acid 30 µg. The zones of inhibition were measured by using the interpretative tables of different zone sizes provided by the manufacturer of the antibiotic discs.

Screening of STEC *E. coli* isolates by PCR assay

Streaking of confirmed STEC *E. coli* isolates was performed on sorbitol MacConkey's agar (SMAC) followed by incubation overnight at 37°C and finally observed for sorbitol fermentation.

Crude DNA obtained from STEC strains using boiling method [14] were presented for PCR screening for presence of *stx1* and *stx2* gene using specific primers as reported by previous co-workers. A 25 µL of final reaction volume of PCR mixture consisted of 12.5 µL master mix (Hi-Media, Mumbai, India), 5 µL (1M) of each primer and 2.5 µL of DNA template. The reaction was carried out at 94°C for 2 min, 35 cycles of 94°C for 60 s, 52°C for 15 s, and 72°C for 1 min, followed by 72°C for 5 min by using specific primers of 5'-ATAAATCGCCATTCGTTGACTAC-3' (forward) and 5'-AGAACGCCCACTGAGATCATC-3' (reverse) for *stx1* and 5'-GGCACTGTCTGAAAC TGCTCC-3' (forward) and 5'-TCGCCAGTTATCT GACATTCTG-3' (reverse) for *stx2* [15].

A 5 µL of reaction mixture along with 1 µL marker DNA (100 bp DNA ladder mix, Hi-Media, Mumbai, India) was mixed separately with 1 µL of 6X gel loading dye (Hi-Media, Mumbai, India) and were gel electrophoresed in 1.5% agarose (Hi-Media, Mumbai, India) at 60 V for 1 hour 20 min. The electrophoresed products were visualized in UV-trans illuminator after staining with ethidium bromide. The amplicon sizes were then compared with the predicted sizes and photographed in Gel Doc EZ imager (Bio-Rad, Hercules, California, USA). To assure the quality of PCR reactions, appropriate negative and positive controls were incorporated into each gel run.

Biofilm assay by tube method

Inoculated isolates on tryptic soy broth with 1% glucose were incubated in shaker incubator broth for 24-48 hrs at 37°C. Thereafter, the broth was decanted, washed with PBS buffer (PH 7.3), dried, and stained with 0.1% crystal violet (CV) followed by washing of excess stain with distilled water and observed for a visible biofilm in the broth-air interphase of the tube.

Results

A total of 83 packed food items were collected and amongst them 4 samples (22.22%) out of 18 cooked items, 10 (33.33%) out of 30 frozen semi cooked items, 13 (54.16%) out of 24 frozen uncooked items, 5 (45.45%) out of 11 uncooked items were observed to be positive for *E. coli*. In total, 32 out of 83 samples were recorded positive for *E. coli*.

All the Gram stained *E. coli* isolates showed Gram-negative bacilli appearing as rods (Figure 1). *E. coli* isolates appeared as pink colored colonies in MacConkey agar (Figure 2) and exhibited characteristic green metallic sheen on Eosin Methylene Blue agar (Figure 3). All the 32 isolates exhibited positive indole, methyl red, negative Voges-Proskauer, urease and citrate. Further, all the isolates were subjected to triple sugar iron (TSI) test where glucose fermentation and lactose producing acid and carbon dioxide were observed and negative H_2S production revealed absence of black precipitates in the agar.

Of the 32 *E. coli* strains isolated from different packed food samples, a 100% resistance towards Ampicillin was observed while on the other hand majority of the isolates exhibited their sensitivity towards Ciprofloxacin (100%), Ceftazidime (100%), and Ofloxacin (100%) whereas Chloramphenicol represented 84.3% susceptibility followed by Nalidixic acid 90.6% and Tetracycline 12.5%.

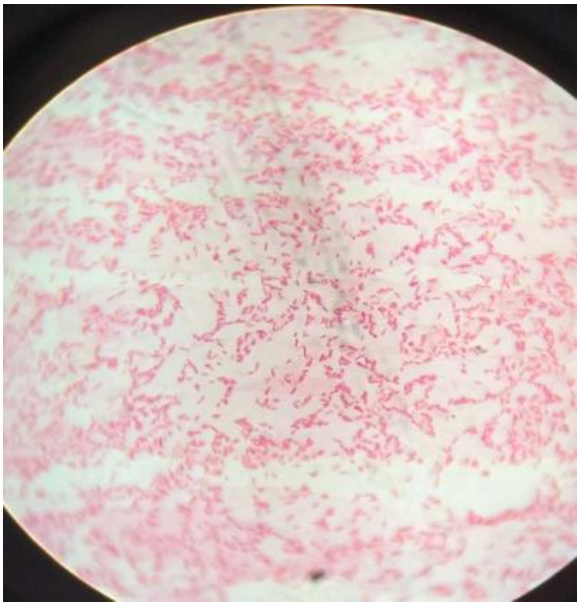

Figure 1. Gram negative *E. coli* rods. (pink)

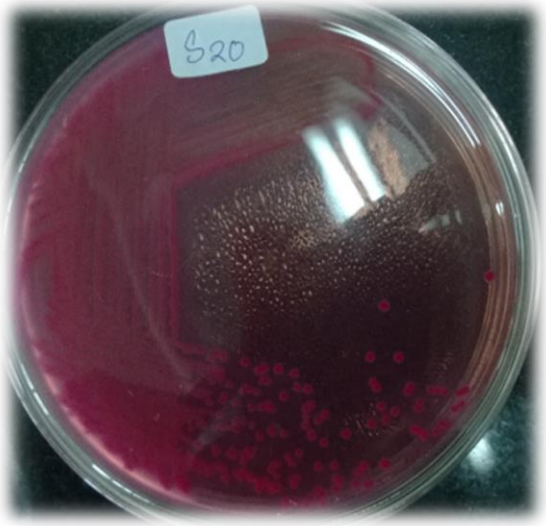

Figure 2. *E. coli* on Macconkey agar showing Colored colonies.

Colony morphology on SMAC revealed that, 11 isolates (34.37%) were Shiga toxin producing (STEC) of the total 32 *E. coli* isolates. All the 11 isolates exhibited colorless colonies, did not ferment sorbitol, and utilized peptone [16].

The amplified product size obtained both in the simplex as well as multiplex PCR (Figure 4) was identical to those predicted from the design of the primers. Thus, the amplified products of 180 bp for *stx1* and 255 bp for *stx2* were obtained. Sorbitol negative and sorbitol positive isolates for the presence of toxin genes were tested where 13 isolates (40.6%) were found to be positive for the presence of Shiga toxin genes, of which 6 isolates represented as *stx1* gene; 2 isolates harbored *stx2* gene while 5 isolates exhibited the presence of both *stx1* and *stx2* genes; while the other isolates in majority harbored *stx1* gene as compared to *stx2* gene.

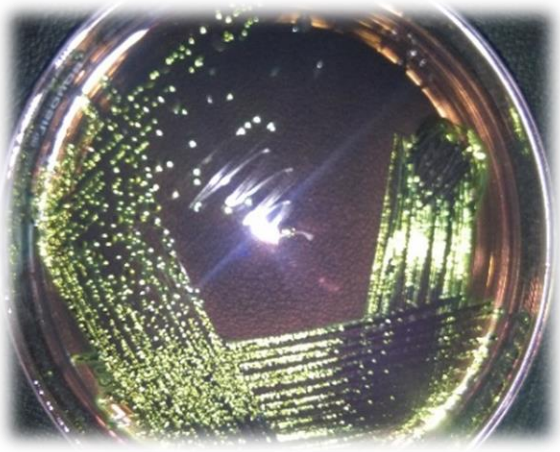

Figure 3. Pure culture of *E. coli* on Eosin methylene blue exhibiting characteristic metallic green sheen.

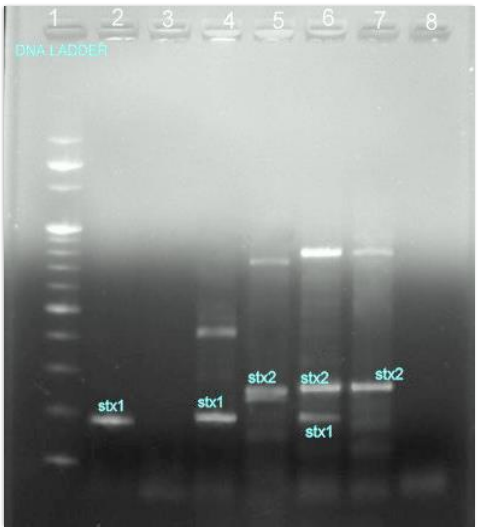

Figure 4. Multiplex PCR using primer *stx1* and *stx2*. **Lane 1**: the DNA ladder (100 bp), **lane 2 and 4:** the amplified product of *stx1* (180 bp), **lane 5 and 7**: the amplified product of *stx2* (255 bp), **lane 6**: the presence of both *stx1* and *stx2*, **lane 8:** the negative control.

Biofilm formation of all the 13 (40.6%) isolates revealed pellicle and distinct film formation at the air-liquid interface (Figure 5) upon 48 hrs of incubation when stained with crystal violet on the surface of the test tube.

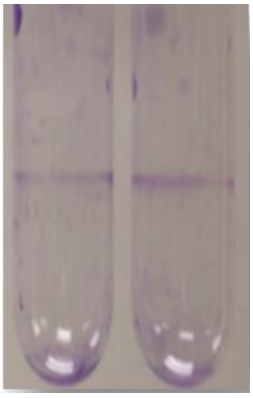

Figure 5. Pellicle formation observed at air broth interphase

Discussion

The presence of organisms in packed food including ready to eat food depicts a deplorable state of poor hygienic and sanitary practices employed in the processing and packaging of these food products [17]. The selection of a reliable medium for the primary isolation of the organism is an essential requirement for the proper understanding of the clinical and epidemiological significance. Thus, in the present study, 83 samples were tested on MacConkey medium and EMB agar to screen *E. coli* isolates in foods obtained from retail markets permitted rapid recovery in presence of very large numbers of competing micro flora [5]. Overall incidence of *E. coli* in different types of packed food was obtained as 22.22% in cooked, 33.33% in frozen semi-cooked, 54.16% in frozen uncooked, and 45.45% in uncooked food samples [18]. Earlier reports by El-Gohany also revealed that foods of animal origin either cooked or uncooked were predominantly contaminated with *E. coli.* [10, 19, 20]. The occurrence of *E. coli* varies from country to country in regard to differences in food and geographical conditions.

Biochemical reactions based on IMViC tests were performed for all the 32 (38.5%) isolates, where typical characteristics of biochemical tests were observed. All isolates resulted as oxidase, Voges-Proskauer, urease and citrate negative and catalase, indole in tryptone broth, methyl red as positive tests; fermented glucose and lactose producing acid and carbon dioxide. Of the total 32 isolates, 11 *E. coli* strains expressed no fermentation of sorbitol when grown on MacConkey Agar with sorbitol (SMAC), thus indicating the presence of O157:H7 serotype amongst the strains. In accordance to several studies, it has been observed that vero cells have been represented with great sensitivity towards *stx* while the cytotoxicity for these cell lines is regarded as one of the gold standard method for confirming the putative *stx* producing isolates [21]. Now-a-days large numbers of bacterial strains have gained resistance against antimicrobial agents due to indiscriminate access to various and easily available antibiotics at the pharmacy stores. Thus, a high alarm regarding public health issues that resistant gene being transferred from the organism in its natural habitat is occurring. In present study antimicrobial susceptibility pattern against the earlier seven mentioned antibiotics was performed where all the 32 isolates exhibited a uniform resistance (100%) towards ampicillin. Majority of the isolates were found to be resistant towards tetracycline (87%) and 100% sensitive towards ciprofloxacin, ceftazidime, and ofloxacin. Further few of the isolates showed resistance towards nalidixic acid (9.3%) and chloramphenicol (15.6%) [14, 22].

However, considering the clinical significance of O157, rapid, specific, and sensitive detection methods are needed to identify toxin producing strains. PCR is a sensitive and specific technique [23]. The use of PCR technology permits the detection of *stx* genes from samples that are microbiologically complex, including samples containing non-viable organisms. In this study PCR based method for detection of genes *stx1* and *stx2* were used to access virulence potential of the isolates. PCR screening revealed the

presence of *stx1* in 5.5% out of 18 vegetarian cooked ready to eat food samples, 5% of 20 veg and 10% of 10 non-veg semi cooked items, 5.5% in 18 veg and 11.7% of 17 non-veg uncooked items. Presence of *stx2* was observed in 5.5% of 18 veg and 5.8% of 17 non-veg uncooked items and finally presence of both *stx1* and *stx2* were observed in 5% in 20 semi cooked veg, 20% of 10 non-veg semi cooked items, and 11.7% of 17 non-veg uncooked items. Similar trend in regard to *stx1* and *stx2* genes was also observed by other researchers [24]. In most of the molecular typing of epidemiological isolates of STEC, the amplification of *stx1* and *stx2* genes with specific primers have been used. In the present study, 15.6% isolates of 83 packed food samples revealed presence of Shiga toxin genes. STEC has shown its capability of biofilm formation which is a colonization factor and it survives on surfaces of steel and also glass utensils. Further it has also been observed that STEC may also form biofilms in different areas of food processing units such as their unit environment, unit floors, food processing equipment (steel, nylon, plastics, glass, aluminum, etc.), walls of the unit, pipes, drains, etc. [25, 26]. In the present study pellicle formation was observed at the air-liquid interface in all 13 Shiga toxin positive isolates [27, 28]. Thus, our results are in concordance with numerous studies regarding surface materials that have significant effects both in bacterial adhesion as well as biofilm formation [29, 30].

Conclusion

Our study highlights the possible health risk associated with the presence of Shiga toxigenic *E. coli* (STEC) in packaged food. The need for stringent measures is required during food production to control cross contamination risks. Since STEC shows capability of biofilm formation which is colonization factor and makes them survive on steel and glass utensils, it puts young children, the elderly, and immune-compromised individuals at risk. The findings of this study suggest the need for a thorough risk assessment on virulence factors of *E. coli* (STEC) particularly isolated from packed food products.

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