Research Article

Molecular epidemiology and prevalence of *Escherichia coli* contamination in fresh vegetables sold at retails in Silchar, Assam, India

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Consumption of fresh vegetables is increasing as people strive to eat healthy diets and benefit from the year-round availability of pre-cut salad items. Salad vegetables belong to convenient, minimally processed food of ready-to-eat type which carry a natural non-pathogenic epiphytic microflora; the majority of which consists of Gram-negative bacteria belonging to *Enterobacteriaceae*. In the present study, 350 raw vegetable samples were investigated for the occurrence of Entero Hemorrhagic *Escherichia coli* (EHEC), and only 44(12.6%) samples were detected to be harboring *E. coli*. The isolates were subjected to PCR detection of EHEC using primers specific for *eaeA*, *ehlyA* and *fliC virulent genes*. According to molecular detection, 9 *E.coli* isolates exhibited presence of *fliC gene*, *ehlyA* and *eaeA gene*. Antibiotic resistances were detected against Piperacillin/tazobactam, Amoxyclav, Levofloxacin, Azetreonam, Ceftriaxone, Imipenem EDTA, Nalidixic acid, Erythromycin, and Kanamycin.

Key Words: Escherichia coli; eaeA; ehlyA; fliC Virulent genes; Antibiotics; DNA Fragments.

INTRODUCTION

Due to multiple benefits associated with a healthy diet, consumption of fresh vegetables and fruits has been increasing over the last decade [1]. However, along with the health benefits, a certain amount of risk is also associated with consumption of fresh vegetables and fruits as they are usually consumed either uncooked or minimally processed to prevent nutrient loss which facilitates the growth of human pathogens [2].

Leafy green vegetables and fruits, an indispensible part of human diet that is usually consumed in raw form are mostly contaminated by *E.coli* [2]. Over the years, there has been a considerable increase in human infections caused due to intake of raw fruits and vegetables as they serve as a transmission pathway for several foodborne pathogens [3,4].

Health problems associated with outbreaks of major food borne diseases resulted in severe economic reduction, internationally [5]. In the present scenario, it turns out to be a global issue and to ascertain that, enough documented data about food borne diseases is available in the literature [6]. *E.coli*, a predominant member of gut flora of human and other animals is usually harmless [7], but some strains can cause intestinal and extra-intestinal diseases and are pathogenic in nature due to acquired virulence factors [8]. Studies indicate survival and ability of *E.coli* to propagate on diverse fruits and vegetables that are minimally processed [9].

There are several reports on infections caused due to consumption of raw fruits and vegetables which are contaminated by *E.coli* O157:H7. A study reported 134 cases of lettuce contamination by *E.coli* in Canada in the year 2008 [10]. Another study reported severe cases which includes 81 samples of lettuce and 199 samples of spinach contamination by *E.coli* that resulted in 3 deaths in US in 2006 [11-13]. Serotype EHEC also called Entero Haemorrhagic *Escherichia coli* is highly pathogenic and has the capability to cause disease even at a very low infection dose of 1-100 cells [14,15]. Shiga toxigenic, Non-Sorbitol Fermenting (NSF) strains of *E.coli* O157:H7 (causative organisms of haemorrhagic colitis and Haemolytic Uremic Syndrome (HUS) caused a large scale outbreak in US during 1980s indicated the emergence of EHEC as human pathogens [16,17]. Potent sources of the contamination of fruits and vegetables before harvesting are manure contaminated by animal feces, dirty water use for irrigation, lack of safety measures followed during process of harvesting, transportation, processing and distribution [3,4,18,19]. Also post-harvest contamination may occur through dirty water used in washing and sprinkling to keep them fresh during marketing process [20] and cross-contamination by means of an infected food-handler [21]. Diarrhoea, Haemorrhagic Colitis (HC), Haemolytic Uremic Syndrome (HUS), Urinary Tract Infections (UTI), septicaemia, and neonatal meningitis are some of the human gastrointestinal and systemic diseases caused by *E.coli* O157:H7 [22]. Ability to colonize, haemolysins and toxin production are virulence characteristics that are encoded by chromosomally located factors (pathogenicity islands, chromosomally inserted bacteriophages) or extra-chromosomal elements (plasmids) which characterize the pathogenicity of *E.coli* [23,24]. Human virulent EHEC strains possess virulence factors such as intimin (coded by *eaeA gene*) that results in formation of attaching and effacing lesions on gastrointestinal epithelial cells, in addition the plasmid encode enterohemolysin toxin (coded by *ehlyA gene*) causing acute haemorrhagic colitis and severe Haemolytic Uraemic Syndrome [25] and *E.coli* flagellar protein (encoded by *fliC gene*) triggers cellular invasion *via* lipid rafts [26].

MATERIALS AND METHODS

Collection of samples

As the study was constrained to Silchar city, samples were collected from various local markets of Silchar in sterilized polythene bags and were processed for *E.coli* isolation within 12-24 h of arrival in the laboratory.

Enrichment of culture

The aseptically collected samples were prewashed with autoclaved water. 1 gram from each sample was added to 10 ml of alkaline peptone water and was allowed to incubate at 37°C for 18-24 hrs. Further, the samples were streaked onto Eosin Methylene Blue agar plates, which results in characteristic greenish-black colonies. The colonies thus obtained [27] were streaked onto MacConkey agar to distinguish isolates of *E.coli* as pink

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coloured colonies [28]. Pure cultures were prepared in Nutrient agar plates [29] and glycerol stocks were stored at -20°C for further analysis [30].

Biochemical identification of *E. coli* isolates

According to the suspected isolates were confirmed by performing a number of biochemical tests namely Oxidase, Catalase, Indole, Methyl red, Voges-Prokauer, Citrate, TSI and Urease tests [27].

Screening for enterohaemorrhagic *E. coli*

The suspected isolates confirmed by biochemical tests were again streaked onto Sorbitol MacConkey agar containing potassium tellurite and cefexime and were incubated overnight at 37°C to confirm the isolates to be EHEC on the basis of their ability to ferment sorbitol. EHEC cannot ferment sorbitol, hence were grown as colorless colonies [31,32].

PCR Amplification

Detection of EHEC was performed using primers selected for amplification of toxin genes i.e. *eae*A [33], *ehly*A [33] and *fli*C *gene* [34]. Genomic DNA was extracted through Phenol Chloroform method [35] and stored at -20°C for PCR reactions. Each reaction mixture of 25 µl contained, 2.5 µl of *Taq* buffer, 0.5 µl dNTPs (200 µm), 1 µl of each primer (10 picomole), 2 µl of template DNA and 1 U of *Taq* DNA Polymerase (Hi Media, Mumbai, India). Nuclease free water was added to make the final volume up to 25 µl. A 32 cycles PCR program was carried out in a thermocycler (Eppendorf). PCR denaturing step was performed at 94°C for 5 min, 94°C for 45 sec, 72°C for 1 min for each toxin gene respectively. The annealing phase was performed at 58°C for 1 min, 56°C for 1 min and 55°C for 45 sec for each gene respectively. The final extension step was performed at 72°C for 8 min. Genes amplified by PCR were run on 1% Agarose gel containing 2 µl ethidium bromide and visualized under Gel Doc EZ imager (Bio-Rad).

Antibiotic susceptibility test

According to the recommendation by the Clinical Laboratory and Standards Institute (CLSI), antibiotic susceptibility testing was performed by Kirby Bauer's disc diffusion method, the test organisms were picked up with sterile cotton swabs and the lawn of bacterial culture was produced over the entire surface of Muller Hinton Agar plates. Aseptic antibiotic discs were placed over the inoculated media surface and were incubated for 24 hrs at 37°C. Post incubation, the plates were examined and the diameters of the clear zones were measured by a ruler in mm. The antibiotics and their potencies used are listed in Table 1.

TABLE 1
Antibiotics and their potencies.

Antibiotic	Potencies (mcg)
Amoxyclav	30
Azetreonam	30
Cefepime	30
Ceftriaxone	30
Erythromycin	15
Imipenem EDTA	10/750
Kanamycin	30
Levofloxacin	5
Nalidixic acid	30
Piperacillin/tazobactam	100/10
Tigecycline	15

RESULTS AND DISCUSSION

Overall incidence of biochemically confirmed *E.coli* 12.6% (n=44) isolates for all types of salad vegetable samples are 18.1% (n=13) for cabbage, 16.7% (n=9) for carrot, 9.6% (n=7) for cucumber, 8.3% (n=3) for tomato, 12.5% (n=5) for coriander, 13.3% (n=6) for radish and 3.3% (n=1) for chilly (Table 2). Maximum incidence is observed in cabbage followed by carrot and radish and minimal is observed in chilly. Low incidence of *E.coli* observed in this study is analogous to the reports of previous studies that obtained 14.3% 17.5%, 18.2%, 19.5%, 21.66% and 26.4% of *E.coli* [36-41].

TABLE 2
Incidence of E. coli in vegetable samples.

SL. No.	Sample material	No. sample	Incidence of *E.coli* in sample
1	Cabbage	72	13
2	Carrot	54	9
3	Cucumber	73	7
4	Tomato	36	3
5	Coriander	40	5
6	Radish	45	6
7	Chilly	30	1
Total	--	350	44

TABLE 3
Frequency distribution of eaeA, ehlyA and fliC in E.coli isolates.

Sl No.	Sample material	No of isolates	Eaea	Percentage distribution (%)	Ehlya	Percentage distribution (%)	Flic	Percentage distribution (%)
1	Cabbage	13	2	15.4	2	15.4	2	15.4
2	Carrot	9	1	11.1	1	11.1	1	11.1
3	Cucumber	7	2	28.6	1	14.3	1	14.3
4	Tomato	3	1	33.3	2	66.6	1	33.3
5	Coriander	5	1	20	2	40	1	20
6	Radish	6	2	33.3	1	16.7	2	33.3
7	Chilly	1	0	0	0	0	1	100
Total	--	44	9	20.24	9	23.44	9	32.5

Studies conducted to evaluate the quality of fresh fruits and vegetables cultivated through organic and conventional farming process had obtained prevalence rates of 0.5%, 2%, 1.6% and 9.7% of *E.coli* respectively [42-44]. Similar study revealed 7%, 12.5%, 16.7%, and 25% of *E.coli* in salad and sprouted seed samples [45-48].

Colony morphology on sorbitol MacConkey agar

Out of the 44 biochemically confirmed *E.coli* isolates, 20.5% (n=9) showed colourless colonies on Sorbitol MacConkey agar. From the colony

morphology on SMAC agar we can presume that 9 *E.coli* isolates are supposedly EHEC.

Polymerase chain reaction

Out of 44 *E.coli* isolates, 9 isolates (20.5%) cannot ferment sorbitol on SMAC (Sorbitol MacConkey) agar which concludes the presence of serotype O157:H7 among the isolates. PCR method was used to detect toxin or virulence potential of the isolates, out of which 9 isolates showed presence of *fliC gene*, 9 isolates showed presence of *ehlyA gene* and 9 isolate showed presence of *eaeA gene* (Figures 1-3). Presence of toxin genes in present study is analogous to reports of previous studies in regards to *eaeA*, *ehlyA* and *fliC genes* with incidence of 6.45% and 7.69% [47-49].

Production of intimin mediated by the *gene eaeA*, an imperative adherence factor that possess ability to synthesize attaching and effacing lesions on intestinal cells [50,51]. Intimin is closely associated with EPEC a strain that releases both Shiga toxins and hemolysins [52]. Presence of the *eaeA gene* in isolates can be potentially considered as enteropathogenic.

Ability of *E.coli* O157 to adhere to leaves and surface of vegetables and fruits is mediated by the filamentous type III secretion system (T3SS) which consists of *EspA* filaments [53]. Also *E.coli* O157 flagella have a significant role in leaf attachment but mutation in flagellin encoded by *fliC* reduces the level of adhesion [54]. All this suggests that *E.coli* O157 uses multiple mechanisms to colonize plants and are well adapted to biosphere.

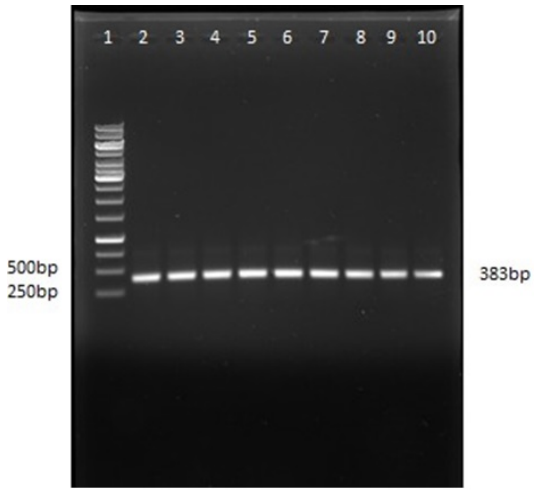

FIGURE 1:: DNA fragments observed with specific primers for *eaeA gene* detection in *E.coli* isolates following PCR. Lane 1: 100 Kb ladder; lane 2-10: isolates samples.

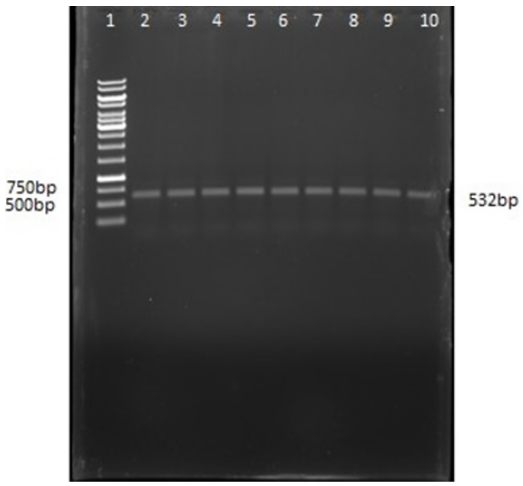

FIGURE 2:: DNA fragments observed with specific primers for *ehlyA gene* detection in *E.coli* isolates following PCR; Lane 1: 1 Kb ladder; lane 2-10: isolates samples.

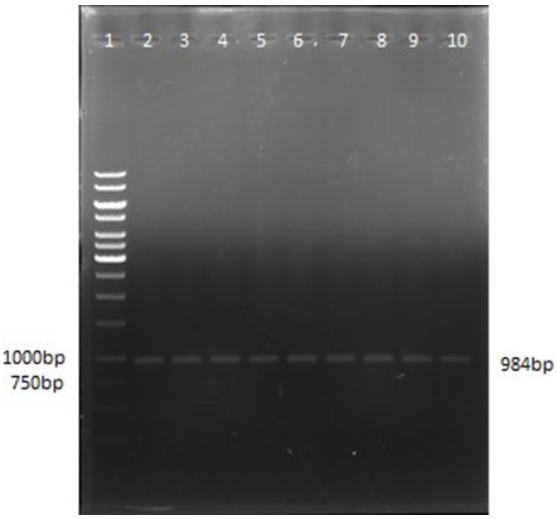

FIGURE 3:: DNA fragments observed with specific primers for fliC gene detection in *E.coli* isolates following PCR; Lane 1: 1 Kb ladder; lane 2-10: isolates samples.

Antibiotic susceptibility pattern of *E.coli* isolates

In the present study, antimicrobial susceptibility pattern against 11 antibiotics were studied for 9 EHEC isolates where it was observed that resistance to the selected drug test ranges from 11.1% to 88.8% for 8 different antibiotics except in case of Cefepime, Tigecycline and Ceftriaxone (Table 4).

TABLE 4
In vitro antimicrobial susceptibility test.

Antibiotic	No of resistant isolates	Percentage of resistance (%)	No. of susceptible isolates	Percentage of susceptibility (%)
Amoxyclav	1	11.1	8	88.8
Azetreonam	1	11.1	8	88.8
Cefepime	0	0	9	100

Ceftriaxone	0	0	9	100
Erythromycin	5	55.5	4	44.4
Imipenem EDTA	2	22.2	7	77.7
Kanamycin	6	66.6	3	33.3
Levofloxacin	2	22.2	7	77.7
Nalidixic acid	3	33.3	6	66.6
Piperacillin/tazobactam	3	33.3	6	66.6
Tigecycline	0	0	9	100

Despite evidence of minimal presence of *E.coli* O157:H7 as observed from this study, microbiological quality is a cause of concern during pre and post-harvest handling and storage of vegetables, considering ability of EHEC to cause infection even at a very low infectious dose [55]. The presence of *E.coli* O157:H7 gives a statement of association with fecal contamination and possibly the indication of presence of other enteric pathogens related with food borne diseases like gastroenteritis and diarrhea [56-58].

E.coli O157:H7 can get internalized in vegetables by utilization of irrigation water and soil contaminated by the organism as recorded from previous studies [59]. Lack of proper microbiological measures undertaken during process of cultivation, harvest, transportation, storage and processing indicates presence of EHEC in vegetables [60,61].

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

CONCLUSIONS

The present study was aimed at isolating *E.coli* from raw vegetables and investigating the occurrence of EHEC in vegetables commonly used as salad items. For this purpose, raw vegetable samples were tested. Out of 350 selected samples 44 *E.coli* isolates were recovered. According to molecular detection, 9 *E.coli* isolates exhibited presence of *fliC*, *ehlyA* and *eaeA gene*. Antimicrobial susceptibility pattern of EHEC isolates reflects resistance to selected drug test ranging from 11.1% to 88.8% for 8 different antibiotics.

E.coli is widely distributed in the environment and are normal commensal found in the intestinal tract. Sources of *E.coli* contamination in food business can be from meat, fresh produce such as vegetables and fruits, unpasteurized milk, water supplies, by direct contact between raw foods and ready-to-eat foods and poor personal hygiene practices. This spread can only be prevented by adhering to strict food safety management procedures. EHEC infection can be asymptomatic or can result in symptoms ranging from abdominal pain, mild diarrhoea and bloody diarrhoea (haemorrhagic colitis) to serious conditions including Haemolytic Uraemic Syndrome (HUS). In a small number of cases, EHEC infection may also develop into Throthrombotic Thrombocytopenic Purpura (TTP). Considering the low infection dose, the presence of *E.coli* O157:H7 in vegetables samples may pose a potential risk for the public health. It can be concluded that application of the Hazard Analysis and Critical Control Points (HACCP) could improve the microbiological safety and quality of these products. Additionally strong interdisciplinary approach is needed in order to answer these questions and to develop safe agricultural systems that will ensure the delivery of safe vegetables to the consumer.

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