



Research Article

Molecular Characterization of *E. coli* Isolated from Raw Vegetable

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ABSTRACT

Vegetables that are commonly consumed in raw form, collected from retail shops viz. coriander leaves, radish, green chilies, cucumber and tomato were tested for contamination with *E. coli*. Out of 80 samples processed, 43 (53.75) percent samples yielded *E. coli*. The highest prevalence of *E. coli* i.e. 86.66 percent was observed in coriander samples followed by 60.00 % in tomato, 53.33 % in reddish, 50.00 % in green chili and 20 % in cucumber samples. The isolates belonged to serogroups O4, O5, O11, O17, O20, O60, O63, O65, O69, O71, O116, O132, O138, O147, three rough strains and 10 untypable. The isolates were subjected to PCR for detection of *mdh*, *clt*, *est*, *stx1* and *stx2* genes. The *mdh* gene was amplified in all 43 isolates while *clt* gene was detected in 30 isolates belonging to six different serogroups viz. O17, O60, O69, O138 and O147 and 7 untypable *E. coli*. The *est* gene was exhibited by 20 isolates belonging to five different serogroups viz. O17, O 60, O69, O138 and 3 rough strain and 5 untypable isolates. Amplification of *stx1* gene was detected in only two isolates, both of O65 serogroup whereas, *stx2* gene was observed in 9 isolates belonging to serogroup O69 and 3 rough strains.

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INTRODUCTION

Use of animal manure for cultivation is a common practice and it is still considered to be better and safer than chemical fertilizers in India. Recently, association between fresh vegetables and outbreaks of food borne infections has led to a greater concern about contamination of vegetables with fecal pathogenic bacteria like *E. coli*, *Vibrio*, *Salmonella*, norovirus, *Shigella*, *Listeria* etc (Mahima et al., 2013) and safety of using animal manures as fertilizer in vegetable production (Pell, 1997; Tauxe et al., 1997). The animal faeces and manure contains number of bacteria that are zoonotic, such as *Escherichia coli*, *Campylobacter* spp. *Salmonella* spp. and *Mycobacterium* spp. (McGarvey et al., 2004; Dhama et al., 2013; Malik et al., 2013; Singh et al., 2013). Therefore, it is imperative to test the vegetables for indicator bacteria that are always present in bovine manure and have survival characteristics comparable to those of fecal enteric pathogens. The *E. coli* is one of the important enteric pathogens among the intestinal microflora of human and warm-blooded mammals. Hence, the present study was designed to study *E. coli* contamination of vegetables that are generally consumed raw or served as salad on dining table using conventional and molecular methods.

MATERIALS AND METHODS

Collection and Preparation of Samples

The study was conducted on the vegetables that are generally consumed raw as salad. Total of 80 samples consisting 15 each of tomato, cucumber, radish and coriander leaves and 20 samples of green chilies were collected from the local retail shops. The samples were collected in sterile polybags and

brought to laboratory. In laboratory, the samples were washed with sterile PBS under sterile conditions and the washings were allowed to stand for ten minutes. The supernatant was discarded; the sediment was collected in sterile glass vials and stored at 4°C until processed for isolation of *E. coli*.

Isolation and Identification of *E. coli*

The enrichment was done by inoculating a loopful of the sediment into MacConkey lactose broth at 37°C for 18hr and then streaked on MacConkey Lactose Agar (MLA). Following 24 hr of incubation at 37°C, the lactose fermenting rose-pink colonies were picked up and streaked onto Eosin Methylene Blue (EMB) agar plates. The samples producing a greenish metallic sheen on EMB plates after 24 hr incubation at 37°C, were transferred on to nutrient agar slants and further processed for biochemical identification as described by Cruickshank et al. (1975). The isolates were identified on the basis of their cultural, morphological and biochemical characters (Edwards and Ewing, 1972) and those preliminarily screened out as *E. coli* were serotyped at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli, H.P., India.

Preparation of DNA Template

The snap chill technique described by Ibenyassine et al. (2007) was used for preparation of DNA template for amplification of virulence genes. For this, 2ml nutrient broth culture of the field isolates was centrifuged at 4,000 rpm for 15 min. The supernatant was discarded; the pellet was suspended in 1ml of sterile triple glass distilled water (TGDW) and centrifuged further for 15min. The resultant pellet was suspended in 1 ml of TE buffer (pH 8.0) and centrifuged at 9,000 rpm for 6 min. The

pellet so obtained was re-suspended in 500 µl of sterile TGDW, boiled for 10 min in a boiling water bath and then rapidly chilled at -20°C. The supernatant was directly used as a template for further amplification of target sequence.

Primers

The published primers (Table 1) based on specific portion of *mdh*, *elt*, *est*, *stx-1* and *stx-2* genes of *E. coli*, were synthesized by Merck (India) and used in the present study.

The simplex PCR assays were carried out in 50µl of reaction mixture containing 25µl of 2X master mix (Merck), 5µl of DNA template, 10 picomoles of forward and reverse primers.

A negative control without template DNA was included in each experiment. PCR cycling was performed in a thermal cycler (Genie TC- 3000) at initial denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 20 sec., annealing at 60°C for 30 sec., extension at 72°C for 30 sec and final extension at 72°C for 5 minutes. The amplified product was electrophoresed on a 1.5% agarose gel in Tris-Borate-EDTA buffer at 80Volts. The gels were stained with ethidium bromide and the bands were visualized under UV gel doc system (UVtech, UK).

Primer pair	Sequence (5' - 3')	Target	PCR amplicon (bp)	Reference
LT3	TATCCTCTCTATATGCACAG	LTI	480	Leong <i>et al.</i> , 1985
LT4	CTGTAGTGGGAGCTGTATA			
STA1	TCTTTCCCTCTTTAGTCAG	STI	166	Ojeniyi <i>et al.</i> , 1994
STA2	ACAGGCCGGATTACAACAAAG			
<i>stx1</i> -F	ATAAATCGCCTATCGTTGACTAC	Shiga toxin I	180	Paton and Paton, 1998
<i>stx1</i> -R	AGAACGCCCACTGAGATCATC			
<i>stx2</i> -F	GGCCTGTCTGAACTGCTCC	Shiga toxin II	255	Paton and Paton, 1998
<i>stx2</i> -R	TCGCCAGTTATCTGACATTCTG			
<i>mdh</i> -1	ACTGAAAGGCAAACAGCCAAG	MDH	392	Hsu and Tsen, 2001
<i>mdh</i> -2	CGTTCGTTCAAATGGCTCAGG			

Table 1:
The oligonucleotide primers used for PCR

RESULT AND DISCUSSION

Out of 80 vegetable samples processed, 43 (53.75 percent) samples yielded *E. coli*. The prevalence of *E. coli* was recorded greatest in coriander samples (86.66 percent) followed by tomatoes (60.00 percent), radish (53.33 percent), green chilis (50.00 percent) and cucumber (20 percent) (Table -2). The distribution of various serotypes among vegetables tested is given in Table -2. The prevalence of *E. coli* in vegetable samples has been reported earlier by many workers (WHO, 1996; CDR, 1997; Buck and Walcott, 2003, Ibenyassine *et al.*, 2007). There are reports that provide the evidence that *E. coli* could be transmitted to vegetables from manure (Wang and Doyle 1998; Natvig *et al.*, 2002), contaminated irrigation water (Fattal *et al.*, 1986; Rai and Tripathi, 2007) and direct contamination from animal and human faeces (Johannesen *et al.*, 2002). Solomon *et al.* (2002) also reported the survival of *E. coli* O157:H7 on lettuce leaves for more than 20 days and concluded that the organism migrated to the internal location in plant tissue and was protected from action of sanitizing agent.

In the present study, *E. coli* isolates of serogroup O4, O5, O11, O17, O20, O60, O63, O69, O71, O116, O132, O138 and O147 were recovered from vegetable washings. Of these, O69 and O147 serogroups were also reported from cases of bovine diarrhea (Diwakar *et al.*, 2008) and serogroups O17, O60, O69, O147 from poultry (Dwivedi *et al.*, 2007; Yadav *et al.*, 2009) from the same study area. Similarly, serogroup O5, O20, O60 and O138 have been reported from diarrheic pigs by Dutta *et al.* (2010). These findings clearly indicate the association of manure with the isolation of *E. coli* from vegetables. Besides this, serogroups O4, O20, O60 and O132 have been reported to be associated with human cases of urinary tract infections, pyelonephritis and renal failure (Johnson, 1991; Blanco *et al.*, 1997; Johnson *et al.*, 2005; Kausar *et al.*, 2009). This indicates that the vegetables sold in domestic market pose serious public health risk as these are injured while peeling, slicing, shredding during salad preparation and usually consumed without heat treatment (Beuchat, 2002). Furthermore, the predominance of

O69 serogroup in this study warrants a special attention to study their role in the development of diarrhea because serogroup O69 is an established shiga toxin producing *E. coli* strain (Griffin and Tauxe, 1991). Serogroup O20 obtained in this study is a well - known human enteric pathogen and has been isolated most frequently from the water in Coimbatore (Grover, 2006).

Conventional culture techniques are universally recognized as gold standard method for diagnosis of *E. coli* but this process is rather lengthy and may last 5-10 days or more. Many foods with short shelf lives would have been consumed by the time the result of analysis becomes available. Hence, the development of simple and rapid assays would enable identification of *E. coli* in contaminated foodstuffs in a timelier manner. Therefore, an attempt was made to detect *E. coli* in vegetable washings using *E. coli mdh* gene primer with minimum amount of time. The PCR amplification of *mdh* gene gave positive result in all 43 isolates and this was in conformity with the findings of Ibenyassine *et al.* (2007). Hsu and Tsen (2001) also used *mdh* gene for direct detection of *E. coli* from milk and water samples. The enzyme malate dehydrogenase from *E. coli* catalyzes the interconversion of malate and oxaloacetate, a critical step in carbohydrate metabolism (Wright and Viola, 2001) and thus, detection of its gene can specifically be used for direct detection of *E. coli* in vegetable washings (Ibenyassine *et al.*, 2007).

The differentiation of *E. coli* pathotypes requires detection of virulence genes either by biological assays or by molecular techniques. Numerous virulence genes were also targeted in present study by simple PCR assay. The presence of *elt* gene was detected in 30 isolates of *E. coli* that belonged to the serogroup O17 (5 strains), O60 (2 strains), O69 (9 strains), O138 (1 strain), O147 (3 strains), 3 rough strains and all ten untypable. The PCR amplification of *est* gene gave positive results in serogroups O17 (5 strains), O60 (2 strains), O69 (9 strains), O138 (1 strain), 3 rough strains and all ten untypable.

Table 2: E coli serotypes associated with raw vegetables

Sample	Total No of samples	E. Coli Positive samples	Positive percentage	Serotypes detected
Green Chili	20	10	50.00	O11,O17,O20,O69 (2),O147(2), Rough, Untypable (2)
Coriander	15	13	86.66	O4,O5,O17,O63,O65, O69(2), O71,O138, Rough, Untypable (3)
Radish	15	08	53.33	O17,O60 (2), O69 (2), O132, Rough, Untypable (1)
Tomato	15	09	60.00	O17 (2), O65,O69 (2), O116, O147, Untypable (2)
Cucumber	15	03	20.00	O69,Untypable (2)
Total	80	43	53.75	

E. coli. In a study, Toma *et al.* (2003) compared the efficacy of simplex and multiplex PCR to categories ETEC strains by targeting *elt* and *est* genes. The present study indicated that ETEC strains belonged to the serogroup O26, O27, O126 and O128. Both the genes were also detected in six untypable strains which were in conformation with our findings. Toni *et al.* (2006) reported presence of *elt* gene in O149 and *est* gene in O8, O20, O64, O101 and O149 serogroups. Presence of these genes indicate the enterotoxigenic nature of these isolates and such ETEC isolates were reported to have the ability to cause profuse, watery diarrhea by release of LT, ST or both enterotoxins (Nataro and Kaper, 1998).

In India, isolation of STEC has been reported from cattle (Pal *et al.*, 1999), sheep (Wani *et al.*, 2004; Bhat *et al.*, 2008), fish (Kumar *et al.*, 2001), human faeces (Khan and Ner, 2002) and piglets (Dutta *et al.*, 2010). But the information related to the prevalence of STEC in vegetables is rare. Hence, in the present study, the isolates were screened for presence of *stx* genes to establish their STEC nature. The *stx1* gene was detected in two isolates both of serogroup O65, however, *stx2* gene was demonstrated in 12 isolates that were representative of O69 (9) and rough strains (3). None of the isolates showed the amplification of both *stx1* and *stx2* genes. However, Dutta *et al.* (2010) reported the presence of *stx1* as well as *stx2* gene in O60 and O138 serogroups isolated from an organized pig farm in Kolkata. Toma *et al.* (2003) have reported *stx* gene in the serogroups O15, O28, O111, O121, O145 and O157 and placed them in STEC category. The STEC strains have been characterized by production of either one or both of the two major types of Stx proteins designated as shiga like toxin-1 (*stx1*toxin) and shiga like toxin-2 (*stx2* toxin) (Rangel and Sparling, 2005) and the production of the later is associated with increased risk of developing Haemolytic Uraemic Syndrome (Boerlin *et al.*, 1999). STEC strains have also been isolated from a number of environmental sources (Rangel and Sparling, 2005) that may result in contamination of vegetables. Detection of *stx1* and *stx2* genes only in two and nine isolates, respectively in present study may also be attributed to loss of virulence genes during subculture as reported by Karch *et al.* (1992).

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