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ORIGINAL ARTICLE

Species specific PCR based detection of *Escherichia coli* from Indian foods

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Abstract *Escherichia coli* is a faecal indicator and certain virotypes are known as pathogens. Therefore, detection and prevention of *E. coli* in food is very important. The existing rapid methods concentrate on detecting the pathogenic *E. coli* instead of total *E. coli* population. Present study evaluates the use of two molecular markers (*uid*A and flanking region of *usp*A) specific for the *E. coli* in combination with microbiological method for confirmation. Majority of the isolates (77%) were positive for both the genes tested. However, 22% of the *E. coli* isolates were positive for any one of the two primer sets [*uid*A (9%) and flanking region of *usp*A (13%)]. High levels of *E. coli* incidences (92% samples) were observed in beef while low occurrence (19% samples) was found in sprouts. Low percentage (7.3%) of *E. coli* isolates was positive for virulence genes tested (*lt*, *ipa*H, *agg*R, *eae*A, *stx*1 and *stx*2). Two isolates were positive for *stx* genes. However, none of the isolates including *stx* positive isolates were *E. coli* 0157:H7. Maximum number of the *E. coli* (44%) isolates was characterized under phylogenetic group B2. This phylogenetic group comprises of extra intestinal and virulent *E. coli* strains.

Keywords *E. coli* · *uid*A · *usp*A · Pathogenic *E. coli* · RT-PCR · Phylogenetic grouping

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Introduction

Escherichia coli is a commensal organism. Many of the *E. coli* strains are versatile pathogens (Kaper et al. 2004). Enterohaemorrhagic *E. coli* (EHEC) strains cause morbidity. Therefore, many epidemiological and molecular pathogenesis studies are carried on EHEC (Strausbaugh 1997). Every year *E. coli* O157:H7 causes 75,000 cases of foodborne disease (Perna et al. 2001). The *E. coli* O157 follows an oral route of infection and about 85% of the *E. coli* O157 infections are food-borne in origin (Friedman et al. 1999, Mead et al. 1999). Contaminated fruits, vegetables and water have been linked to *E. coli* O157:H7 outbreaks (Ferens and Hovde 2011).

A few rapid methods are reported for identification of *E. coli*. Chen and Griffiths (1998) have proposed flanking region of *uspA* gene for the detection of *E. coli*. Bej et al. (1991) have shown that *uidA* gene sequence is unique to *E. coli*. The *uidA* and *tuf* gene was used by Maheux et al. (2009) for the detection of *E. coli* and *Shigella* strains. However, none of these methods are extensively tested in the field.

Since *E. coli* gained prominence as a pathogen, studies were carried out to characterize these isolates with respect to virulence genes. Phylogenetic grouping of food and clinical strains of *E. coli* would help to understand the distribution of such strains in the environment. Clermont et al. (2000) classified *E. coli* into 4 phylogenetic groups (A, B1, B2 and D groups) based on the presence and absence of the 3 unique regions on *E. coli* genome.

Nevertheless, detection and confirmation of the *E. coli* is a time consuming process. Extensive work has been carried out previously to detect the presence of pathogenic *E. coli* serovars. However, incidence of all strains of *E. coli* in food and their characterization is lacking. This study

focuses on the detection and confirmation of *E. coli* in Indian food samples using conventional microbiological method (Anonymous 2014) in combination with molecular method. This study also reports the molecular characterization of these *E. coli* isolates with respect to the incidence of virulence genes and phylogenetic grouping.

Materials and methods

Sample collection and enrichment

One hundred and seventy food samples comprising of chicken (34), mutton (34), beef (22) sprouts including green gram, moth beans, field beans, chick pea, etc. (26), marine fish (28) and pork (26) were analysed for presence of the *E. coli* from Mumbai, India. All the samples were collected from the local retailers and received at the laboratory within 2 h of purchase. The samples were kept at 4 °C till the analysis.

All microbiological media and chemicals were obtained from Hi-Media (Mumbai, India). Primers were obtained from Integrated DNA technologies (California, US) and all PCR reagents were obtained from Finzyme (San Diego, US).

All the samples were processed as described in US Food and Drug Administration Bacteriological Analytical Manual (Anonymous 2014) with some modifications. In brief, the samples (25 g) were homogenised in 225 mL brain heart infusion (BHI) broth. After incubation at 35 °C for 3 h in static condition, the homogenate was transferred in a 225 mL of double strength tryptone phosphate broth (TPB) for a further incubation of 20 h at 44 °C. A loop full (10 µL) of homogenate was plated on the selective media, MacConkey agar and L-Eosin Methylene Blue (L-EMB) agar, in duplicate. These plates were incubated for 20 h at 35 °C. Typical colonies or in the absence of typical colonies, two atypical colonies from each plate were re-streaked on MacConkey agar and L-EMB agar.

Molecular confirmation of the presumptive positive *E. coli*

DNA was extracted by boiling a single colony of the presumptive positive cultures in 100 µL of molecular grade water for 5 min, followed by centrifugation (10,000*g* for 5 min) and 1 µL of the supernatant was used as template DNA for PCR. The confirmation of *E. coli* was carried out by two PCR reactions. The flanking region of *uspA* (the universal stress protein) amplification (Table S1) was carried out as described by Chen and Griffiths (1998) and *uidA* (β-glucuronidase) gene amplification (Table S1) was performed as described by Heijnen and Medema (2006). A multiplex PCR was carried out using *uidA* and flanking region of *uspA* primers by the following program; 94 °C for 5 min, 35 cycles of annealing at 55.2 °C for 10 s, extension at 72 °C for 1 min, denaturation at 94 °C for 10 s; and a final extension of 72 °C for 10 min in the thermal cycler (Eppendorf, Germany).The amplified DNA (5 µL per lane) was run on a 2% agarose gel at constant 70 V. The gel was visualized under UV light.

Biochemical and serology tests

Biochemical tests were carried out for the untypable *E. coli* strains. Also the isolates that were positive for amplification by one set of primers were confirmed as the *E. coli* by the above tests using the US-FDA (Anonymous 2014). Major biochemical tests done for evaluating *E. coli* were IMViC (Indole, Methyl red, Vogous prosker and citrate) tests.

Serology with respect to O and H antigen was carried out at National Centre for *Salmonella* and *Escherichia*, Central Research Institute, Kasauli, India of the isolates that were positive for the presence of virulence genes (Table 2).

Determination of virulent variants

The isolates were examined for *stx1* and *stx2*, to identify the EHEC (Enterohemorrhagic *E. coli*) strains; *eaeA*, intimin for EPEC (Enteropathogenic *E. coli*) attaching and effacing; *aggR* for EAEC (Enteroaggregative *E. coli*); *ipaH* for EIEC (Enteroinvasive *E. coli*); *lt* for heat labile; *st* for heat stable toxin; for ETEC (Enterotoxigenic *E. coli*) and *daaD* for DAEC (Diffusely adherent *E. coli*) using the primers specific to the above genes as described by Guion et al. (2008). The presence of *stx1* and *stx2* genes was further confirmed using the probe based real time PCR as described by US-FDA (Anonymous 2014). All the *E. coli* isolates were screened for the presence of +93 *uid*A gene (Anonymous 2014) specific for the O157:H7 serotype by real time PCR (Anonymous 2014) and also plated on MUG (4-methylumbelliferyl β-D-glucuronide) sorbitol agar according to the US-FDA (Anonymous 2014).

E. coli phylogenetic grouping

The *E. coli* phylogenetic grouping was determined by the amplification of DNA targets using a single multiplex PCR as described by Clermont et al. (2000). The genomic DNA isolated from each *E. coli* strain was used for amplification of the *chuA*, *yjaA* and DNA fragment TSPE4.C2. The amplified DNA (5 µL per lane) was run on a 2% agarose gel at constant 70 V. The gel was visualized under UV light. Based on the presence or absence of 3 DNA markers

(*chuA*, *yjaA* and DNA fragment TSPE4.C2) isolates were assigned to specific phylogenetic groups as per the scheme proposed by Clermont et al. (2000).

Results and discussion

uidA and *uspA* as marker genes for the confirmation of *E. coli* from food samples

A total of 448 presumptive positive isolates of *E. coli* were isolated from food samples by biochemical method. Typical colonies from the selective plates were confirmed by two *E. coli* specific primers for the marker gene (*usp*A and *uid*A). Isolates which were positive for both the sets of primers and either of the two primers were considered as *E. coli*. 149 isolates (79%) from the food samples tested were positive for the presence of the marker genes, *uidA* and flanking regions of *usp*A (Fig S2). However, we observed that 43 (22%) of the *E. coli* isolates were positive for any one of the two primer sets [*uid*A (9%) and flanking region of *usp*A (13%)]. The *uid*A gene has been shown to be very specific to *E. coli*; however, primers specific to this region also amplifies few species of *Shigella* (Bej et al. 1991). Heijnen and Medema (2006) have shown that modified primer set for *uid*A detected not only all *E. coli* isolates tested, but also 54% of the *Shigella* species. To address this shortcoming of *uid*A primers, we included *E. coli* specific primer set for flanking region of *usp*A (Chen and Griffiths 1998). Twenty two percent of the *E. coli* isolates which were positive for either one of the two *E. coli* specific primers were further confirmed by additional biochemical tests and were found to be positive for *E. coli*. Eight per cent of atypical colonies on L-EMB and MacConkey plates were positive for *E. coli* by PCR. Thus, atypical colonies on selective plate should be tested for *E. coli*. Although, molecular confirmation is better than phenotypic detection, there are possibilities that molecular detection based on one marker gene may give false negative results. Hence, the present study suggests the use of multiple markers for confirmation of *E. coli*. These two sets of primers can be run as multiplex PCR (Data not shown). In recent work Molina et al. (2015) have shown that *yai*O and *lacZ3* primers are unique to *E. coli*. However, these primers are tested with limited number of samples and not tested in field. PCR based confirmation of typical and atypical colonies obtained on selective plate for *E. coli* would increase speed and accuracy of the identification (Fig S1).

Prevalence of *E. coli* in Indian food samples

E. coli is a faecal coliform used as an indicator of human enteric pathogens (Orskov et al. 1987). Therefore, the occurrence of *E. coli* in high percentage of food samples indicates poor hygiene of the food samples. More than half of the food samples tested was positive for *E. coli* (Table 1). High level (91%) incidence of *E. coli* was observed in beef samples and a comparatively low occurrence in the sprouts (19%) (Table 1). There are many reports of incidence of *E. coli* in food from different parts of the world. In a study from Mexico street food, 44% of the samples were positive for *E. coli* (Estrada-Garci et al. 2004). Li et al. (2004) have shown that 38% of bison carcasses are contaminated with *E. coli*. There is a possibility of non-pathogenic *E. coli* may acquire virulence genes by horizontal gene transfer and results in emergence of virulent strains (Ochman et al. 2000).

Incidence of virulence genes in the *E. coli*

All 192 isolates were characterized with respect to virulence genes and occurrence of the O157:H7 serotype using rapid and multiplex PCR method (Guion et al. 2008). In the current study, 7.3% of the *E. coli* isolates were positive for virulence genes (Table 1). All the *E. coli* isolates from sprout samples were negative for virulence genes tested. The *E. coli* isolated from mutton showed high incidence of virulence genes (21%) (Table 2). Previous report has shown the incidence of pathogenic *E. coli* in beef, chicken, pork and other animal meats (Magwedere et al. 2013). Isolates which showed presence of virulence genes were serotyped; 4 isolates belonged to O124, contained *lt*, *aggR* and *eaeA* genes, 2 isolates which showed presence of *stx* genes were categorized as *E. coli* rough strain and rest of the isolates were characterized as untypable *E. coli* (UT *E. coli*) (Table 2); however, isolates characterized as UT *E. coli* were positive for both the *E. coli* marker genes (flanking region of *uspA* and *uidA*) and positive for IMViC tests. *E. coli* O157:H7 was absent in all the food samples tested. Few studies have reported the incidence of *E. coli* O157:H7 in food samples from India (Verma et al. 2013). In an epidemiological survey of 17,000 *E. coli* isolates from food and clinical samples, low incidence of *E. coli* O157 was observed (Sehgal et al. 2008). Bindu and Krishnaiah (2010) have shown the presence of virulence genes in *E. coli* with special reference to *stx1* and *stx2*. In the current study, toxin genes *stx1* and *stx2* were found in one *E. coli* isolate from the mutton sample and the presence of *stx1* was detected in one isolate from the beef sample. The incidence of both *stx1* and *stx2* gene was not observed in any of the *E. coli* isolate.

Similar to our results, Sheikh et al. (2013) observed low incidence of *stx* genes in *E. coli* isolates of India.

Distribution of phylogenetic groups in *E. coli*

In this study, 192 *E. coli* isolates obtained from different food samples were assigned to four phylogenetic groups

Table 1 Incidence of non-pathogenic and pathogenic *E. coli* in different food samples

Samples	No. of samples	No. of samples positive for *E. coli*	No. of *E. coli* isolates	No. of pathogenic/virulent *E. coli*
Chicken (C)	34	20 (59%)	34	1
Mutton (M)	34	18 (52%)	32	7
Beef (B)	22	20 (91%)	36	3
Pork (P)	26	22 (84%)	45	2
Fish (F)	28	21 (75%)	33	1
Sprouts (S)	26	5 (19%)	12	0
Total	170	106 (62%)	192	14 (7.3%)

Table 2 Characterization of pathogenic *E. coli* based on different virulence genes and phylogenetic groups

Isolates	*eaeA*	*aggR*	*ipaH*	*lt*	*st*	*stx1*	*stx2*	*daaD*	Phylogeny group	Type of pathogen[a]	Serotype
C1	−	−	−	+	−	−	−	−	A	ETEC	UT
M1	−	−	−	−	−	+	+	−	B1	STEC	Rough
M2	−	−	+	−	−	−	−	−	B2	EIEC	UT
M3	−	−	−	+	−	−	−	−	B2	ETEC	UT
M4	−	−	−	+	−	−	−	−	A	ETEC	O124
M5	−	−	−	+	−	−	−	−	A	ETEC	Rough
M6	−	+	−	−	−	−	−	−	B1	EAEC	O9
M7	−	+	−	−	−	−	−	−	B1	EAEC	Rough
F1	−	−	−	+	−	−	−	−	B1	ETEC	UT
B1	−	−	−	−	−	+	−	−	B2	STEC	UT
B2	−	+	−	−	−	−	−	−	A	EAEC	O124
B3	+	−	−	−	−	−	−	−	B2	EPEC	O124
P1	−	+	−	−	−	−	−	−	B2	EAEC	O124
P2	−	−	+	−	−	−	−	−	B1	EIEC	UT

ETEC strains are *st*and/or *lt* positive; STEC strains are *stx1* and/or *stx2* positive; EPEC strains are *eaeA* positive; EAEC strains are *aggR* positive; EIEC strains are *ipaH* positive (Guion et al. 2008); '+', positive, '−', negative, UT, untypable

[a] Classification of pathogenic *E. coli* is done according to Guion et al. (2008)

(A, B1, B2 and D). Maximum number of the *E. coli* isolates (44%) were characterized under group B2. This is a significant observation because majority of the extra intestinal and virulent *E. coli* were classified under phylogenetic group B2 (Bingen et al. 1998). This result is consistent with the previous study by Rugeles et al. (2010). The group B2 is the most prevalent phylogenetic group in meat samples. Three percent of the isolates belonged to group D which also represents the virulent *E. coli* (Bingen et al. 1998). Thus, 47% of the isolates obtained from these food samples belong to two phylogenetic groups of *E. coli* which represent pathogenic *E. coli*. In this study, the phylogenetic groups A and B1 constituted of 16% and 29%, respectively. Group B2 commonly occurred among pork (53%), beef (72%), mutton (47%) and fish (33%). Chicken (18%) and sprouts (25%) mainly showed the presence of D group. Virulence gene positive *E. coli* isolates (7.3%) belonged to the B1, B2 and A group. Group B2 was the major phylogenetic group in all food samples of animal origin. This observation suggests that major reservoir of pathogenic *E. coli* are animals.

Conclusion

The combination of molecular and biochemical confirmation of *E. coli* is rapid and specific as compared to conventional identification method. The pair of DNA marker (*uspA* and *uidA*) is more accurate for the confirmation of *E. coli* than any one single DNA marker. In this study, beef

samples showed the high incidence of *E. coli*. High percentages of food samples were positive for *E. coli*. 7% of *E. coli* isolates were positive for virulence genes tested. Two *E. coli* isolates were positive for *stx* genes. However, all the samples tested were free of *E. coli* O157:H7. More than one-third of isolates were categorized under phylogenetic group B2 which is known to contain extra intestinal and virulent *E. coli* strains.

Compliance with ethical standards

Conflict of interest There is no conflict of interest to declare.

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