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Application of multiplex PCR assay based on *uidR* and *fliCH7* genes for detection of *Escherichia coli* O157:H7 in milk

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A highly sensitive and specific multiplex PCR assay has been developed to detect the presence of *Escherichia coli* O157:H7 from naturally contaminated raw milk samples within 10 h. The primers explored in the assay were targeted against the *uidR* gene specific for all types of *E. coli* and the *fliCH7* gene specific for the h7 flagellar antigen of *E. coli* O157:H7. The multiplex PCR assay developed was found to be highly specific as it produced PCR products of 152 bp (*E. coli* specific) and 625 bp (*E. coli* O157:H7 specific). The assay was tested for its specificity against different serotypes of *E. coli* as well as other pathogenic strains like *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Staphylococcus aureus*, *Lactobacillus* and *Lactococcus* etc. When this multiplex PCR assay was directly applied to 24 raw milk samples collected from different sources, *E. coli* O157:H7 could be detected in one of the milk samples without 4 h enrichment in CT-SMAC broth and three samples after 4 h enrichment in CT-SMAC broth. However, all the pasteurized milk samples gave a negative signal for this organism.

Key Words—*Escherichia coli* O157:H7; *fliCH7*; multiplex PCR; raw milk; *uidR*

Introduction

Escherichia coli O157:H7 is a high risk pathogen of considerable public health significance because of its involvement with serious human illnesses including Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TPP), which have been reported with increasing frequency ever since this organism was first reported in 1982 (Ferens and Hovde, 2011; Jay et al., 2004; Wells et al., 1983; Witold et al., 2011). Transmission of this pathogen occurs primarily in ground beef (Griffin

and Tauxe, 1991; Savoye et al., 2011). However, other foods including raw and pasteurized milk, yoghurt and cheese have also been epidemiologically implicated (Chapman et al., 1993). Raw milk was first recognized as a vehicle of transmission of *E. coli* O157:H7 in 1986 (Martin et al., 1986; Oberst et al., 1998) when children from Wisconsin, USA, developed HC and HUS after drinking raw milk from dairy farms. Since then, there have been several reports of outbreaks associated with this pathogen following consumption of raw milk (Borczyk et al., 1987; Doyle, 1991; Duncan et al., 1987; Karna et al., 2007; Mai et al., 1987). Yet another *E. coli* O157:H7 outbreak associated with consumption of pasteurized milk raised considerable concern about the milk-borne transmission of this pathogen. The organism could be isolated from milk handling pipes and bottling machine in a dairy plant, indicating that inadequate pasteurization or post-pasteurization con-

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tamination were responsible for the outbreak (Morgan et al., 1993). Later, Upton and Coia (1994) reported a large outbreak of *E. coli* O157:H7 infection in the UK due to pasteurized milk. Seghal et al. (2008) presented an epidemiological survey of *E. coli* O157:H7 in different regions of India. They reported that a significantly high percentage of *E. coli* O157:H7 was isolated from milk and milk products. In view of its considerable public health significance and involvement in gastroenteritis outbreaks, timely detection of this organism in foods, particularly in milk and other dairy foods, is extremely important to assess the safety of the products. Several methods have been developed for the detection and isolation of *E. coli* O157:H7 from foods and other samples. Molecular approaches based on PCR assays have also been explored in order to develop sensitive, specific and rapid methods for confirming the presence/absence of *E. coli* O157:H7. In this context, although a lot of efforts have been made by different investigators to develop PCR-based assays targeted against different genes for detection of *E. coli* O157:H7 (Cadirci et al., 2010; Cocolin et al., 2000; Gooding and Choudary, 1997; Gordillo et al., 2011; Kawasaki et al., 2010; Park et al., 2011; Paton and Paton, 1998; Rice, 2009), the efficacy of these techniques has been tested with food samples artificially spiked with the target organism. Application of such PCR-based techniques has not yet been efficiently and extensively investigated for detection of *E. coli* O157:H7 in naturally contaminated dairy foods. Hence, the development of PCR-based techniques for detection of a high-risk pathogen like *E. coli* O157:H7 in perishable dairy products could be extremely valuable and relevant. In the present investigation, a multiplex PCR assay based on *uidR* and *fliCH7* genes, specific for *E. coli* and *E. coli* O157:H7, respectively, was developed and applied for the screening of raw and pasteurized milk samples.

Materials and Methods

Bacterial cultures and their maintenance. The bacterial cultures used in this investigation included both pathogenic and non-pathogenic strains of *E. coli* along with *E. coli* O157:H7 as well as other non-*E. coli* cultures. *E. coli* O157:H7, EHEC 933, EPEC O11ab:NM, EAaggEC, H10407 were procured from Christian Medical College, Vellore, India. Another strain of *E. coli* O157:H7 was also procured from NIZO, The Nether-

lands. *E. coli* ATCC and NCTC strains were obtained from All India Institute of Medical Sciences, New Delhi, India. The other non-pathogenic *E. coli* cultures, namely K-12 and DH5 α , and non-*E. coli* pathogens, viz. *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas fragi*, and *Proteus vulgaris*, as well as lactic cultures, namely *Lactobacillus helveticus* and *L. delbrueckii* subsp. *bulgaricus* RTS, were procured from National Centre for Dairy Cultures, National Dairy Research Institute, Karnal, India. *E. coli* E-10 was obtained from Netherland Institute of Dairy Organisms, The Netherlands.

All the *E. coli* and non-*E. coli* cultures were propagated either in Brain Heart Infusion (BHI, Difco) or Luria-Bertani (LB) broth at 37°C for 18–24 h. *E. coli* O157:H7 stock cultures were also checked by streaking on CT-SMAC (Cefixime Tellurite Sorbitol MacConkey's agar) (March and Ratnam, 1986) as well as on Chrom agar (Dynal, UK). The cultures were preserved on BHI/ LB agar slants/stabs, and stored in a refrigerator or as glycerol stocks stored at –70°C for further use. The lactic acid bacteria used in the study were preserved in litmus milk tubes and cultured periodically.

Preparation of template DNA

Broth cultures: The bacterial strains of *E. coli* O157:H7, *E. coli* or non-*E. coli* were cultured in LB broth and incubated at 37°C for 18–24 h. The template DNA from the broth cultures was prepared by the boiling method. The boiled cell lysate was prepared by heating the bacterial suspension in distilled water for 10 min in a boiling water bath and then centrifuging for 5 min at 10,000 rpm to pellet the cell debris. This cell lysate was used as a template for pure culture preparations. The DNA was also extracted from pure broth cultures by following the method of Pospiech and Neikmann (1995). The cells were harvested from 1 ml of overnight-grown cultures of the test *E. coli* cultures and resuspended in 0.5 ml of SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris), and lysozyme buffer (25 mM Tris, lysozyme 10 mg, 5 M NaCl, pH 8.0) was added at a concentration of 1 mg/ml, followed by incubation at 37°C for 1 h. The subsequent step was the addition of 1/10 volume of 10% SDS and 0.5 mg/ml of proteinase K, and incubation further continued for 2 h at 55°C. One third volume of 5 M NaCl and 1 volume of chloroform were added and incubated at room temperature for 30 min with frequent inversions. The samples were centrifuged, the aqueous phase was transferred to a

new tube and the DNA was precipitated by adding 1 volume of isopropanol or 2 volumes of ethanol. The DNA was pelleted, dried and dissolved in TER buffer (10 mM Tris-Cl, 1 mM EDTA, 10 µg/ml of RNase A, pH 7.5). The sensitivity of the PCR assay was determined by using different concentrations (500 to 1 ng) of *E. coli* DNA as a template. For determining the sensitivity in terms of CFU, LB broth was inoculated with *E. coli* O157:H7 at the level of 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10 CFU/ml, and template DNA from each sample was extracted as described above.

Spiked skim milk samples: Skim milk was prepared by dissolving 11 g of non-fat dry skim milk powder (NFDM) in 100 ml distilled water, autoclaving at 121°C for 15 min and dispensing in aliquots of 1 ml each in sterilized eppendorf tubes. The aliquots of sterilized skim milk were then separately inoculated with *E. coli* O157:H7 at different levels ranging from 10^6 to 10 CFU/ml. The DNA from spiked milk samples before and after 4 h enrichment in MacConkey's broth was then extracted by following the 'NDRI method' previously developed in our laboratory (Suresh, 1999). In brief, equal amounts of *n*-propanol, 25% ammonia, petroleum ether and diethyl ether were added to milk samples. After centrifugation, the fat disk was removed and the supernatant transferred to a fresh eppendorf tube. One milliliter aliquots from the samples were then centrifuged and the pellet dissolved in 400 µl of GTC buffer (5.88 M guanidinium isothiocyanate, 100 mM Tris HCl, pH 7.0) and 400 µl of phenol saturated with TE (pH 8.0). Chloroform extraction was then carried out, and DNA was pelleted as described above.

Raw and pasteurized milk samples: The raw and pasteurized milk samples were collected aseptically from the local market. The samples were first pre-enriched for 4 h in CT-SMAC broth. Template DNA was extracted from 10 ml aliquots of milk samples before and after enrichment in CT-SMAC using the above described 'NDRI' method. The samples were also analyzed microbiologically both before and after enrichment.

PCR amplification. The PCR amplification for detection of *E. coli* O157:H7 was performed using a PTC-100 Thermal Cycler (MJ Research, USA). Oligonucleotide primers were custom synthesized (Bangalore Genei, Bangalore, India). The primer pairs, namely *uidR* [URL-301: 5'TGT TAC GTC CTG TAG AAA GCC C3' and UAR-432: 5'AAA ACT GCC TGG CAC AGC AAT3' (Bej et al., 1991)] and *fliCH7* [*fliCH7F*: 5'GCG

CTG TCG AGT TCT ATC GAG C3' and *fliCH7R*: 5'CAA CGG TGA CTT TAT CGC CAT TCC3' (Gannon et al., 1997)] targeted against β -glucuronidase gene of *E. coli* and flagellar antigen h7 of *E. coli* O157:H7, respectively were used in the assay. The PCR assay was performed in a 25 µl reaction volume using 0.5 ml PCR tubes. The reaction mixture comprised 100 ng of template DNA, 1×PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM MgCl₂), 0.2 µM (each) primer set, 0.2 mM (each) dNTPs and 1.5 U of *Taq* DNA Polymerase (Boehringer Mannheim, Germany). The PCR cycling programming was carried out according to the temperature stringency conditions of the primer pairs used, and the optimized PCR cycling conditions for each set of primers are given in RESULTS AND DISCUSSION. Each cycle was repeated 35 times and the final cycle was followed by an extension of 10 min at 72°C.

Analysis of PCR products. The PCR products were electrophoresed on 2.0% agarose gel containing 0.5 µg of ethidium bromide. The molecular size marker consisted of a 100 bp DNA ladder comprising 100 to 1,000 bp bands (Bangalore Genei, Bangalore, India).

Results and Discussion

The main focus of this study was to develop a PCR assay which could be directly applied to milk samples in order to detect the presence of *E. coli* O157:H7. Hence, we initially tried a number of primers targeted against different gene sequences [*rfbE0* (Desmarchelier et al., 1998); *stx1* and *stx2* (Paton and Paton, 1998); VT (Nagano et al., 1998); *eaeA*_{O157} and *fliCH7* (Gannon et al., 1997)] to test their efficacy and specificity. After thorough evaluation (data not shown), the primer pair *fliCH7* was found to be highly specific for verotoxigenic *E. coli* O157:H7. The *uidR* set of primers based on β -glucuronidase gene present in all *E. coli* strains was combined with *fliCH7* to develop a multiplex PCR that could detect *E. coli* and *E. coli* O157:H7 as they produce different-sized PCR specific products of 152 and 625 bp, respectively. However, before combining the two sets of primers for developing a multiplex PCR, they were individually tested for their sensitivity and specificity in respective PCR assays.

PCR assay using *uidR*-based primers

All *E. coli* are known to produce β -glucuronidase, which is encoded by 'uid' gene; hence, we explored a set of primers targeted against the *uidR* gene in our

PCR assay. The optimized PCR parameters for the assay were found to include an initial denaturation for 5 min at 95°C followed by 25 cycles of 1 min each of denaturation at 94°C, annealing at 59°C and extension at 50°C with a subsequent final extension at 72°C for 5 min. The *uidR*-specific PCR product of 152 bp was observed in all *E. coli* cultures. The specificity of this primer set was also ascertained against other non-*E. coli* strains. The only non-*E. coli* strain that produced this amplified product was *Shigella dysenteriae*, due to the genetic relatedness of this organism. The results in this regard are consistent with the observations of Bej et al. (1991), who developed a PCR-probe assay based on amplifying the regions of the 'uid' gene that codes for β -glucuronidase, and expression of which forms the basis for fecal coliform detection by the commercially available colilert test. They used two sets of primers encoding the *uidA* and *uidR* genes for PCR assay. However, the assay demonstrated that the target *uidA* gene is present in *E. coli* and *Shigella* species but no other coliform or non-enteric bacteria, indicating a high degree of homology between the two bacterial species. In our study, the *uidR* PCR assay was found to be very sensitive as it produced a 152 bp PCR product even with 1 ng of template DNA. The results in this regard are more or less comparable with those of Bej et al. (1991), who also found the sensitivity of their PCR assay to be 10 fg equivalent to the detection of 1–2 CFU.

PCR assay using *fliCH7*-based primers

Primers based on flagellar antigen h7 specific for *E. coli* O157:H7 were also tried. The amplification parameters included 35 cycles each of denaturation at 94°C/15 s, annealing at 65°C/15 s and extension at 72°C/75 s, followed by a final extension at 72°C for 5 min. The PCR assay was found to be highly specific for *E. coli* O157:H7 as it yielded a product of 625 bp. However, the 625 bp product could not be obtained with any other *E. coli* strains tested. Instead, some non-specific products were observed in ATCC and NCTC strains indicating that *fliCH7*-based primers were quite unique for *E. coli* O157:H7. The specificity of this set of primers was also tested against other non-*E. coli* strains, namely *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Proteus vulgaris*, as well as against lactic acid bacteria, namely *Lactococcus lactis* subsp. *lactis* and *L. delbruekii* subsp. *bulgaricus*. However,

none of these cultures could produce the specific 625 bp product. The sensitivity of the PCR assay was also tested and it was found to amplify as little as 1 ng of template DNA. A comparative analysis of our results indicated that *fliCH7*-based primers are the ideal candidates for development of a PCR assay for the detection of *E. coli* O157:H7. The results obtained in this regard are similar to those of Gannon et al. (1997) and Nagano et al. (1998), who also reported that a primer set based on the *fliCH7* sequence amplified specific DNA fragments from all *E. coli* H7 strains but did not amplify fragments from any other bacterial strains. Mukhopadhyay and Mukhopadhyay (2007) reported the sensitivity of the multiplex PCR for simultaneous detection of *E. coli* O157:H7 and *Listeria monocytogenes* using *fliCH7* and *iap* sets of primers, respectively. In the unenriched sample, the detection limit of multiplex PCR using crude DNA template was found to be 10¹ CFU. However, in the post-enriched sample only *E. coli* O157:H7 specific products across all levels were seen.

Multiplex PCR assay using *uidR*- and *fliCH7*-based primers

After evaluating the performance of *uidR*- and *fliCH7*-based sets of primers in respective PCR assays, efforts were then made to combine these primer sets with the objective of developing a multiplex PCR. The multiplex PCR assay conditions were standardized by using different annealing temperatures to get specific amplification of both *uidR* and *fliCH7* simultaneously by using template DNA from pure cultures of *E. coli* K-12 and *E. coli* O157:H7. Five different programmes with varying annealing and extension temperatures were tried (details are given in Table 1). The specific PCR products of 152 and 625 bp were obtained with programme EcUH6365 comprising 35 cycles each of an annealing and extension temperature. Non-specific products were not detected in any of the samples by using these PCR parameters. However, with other programmes, several non-specific bands could be invariably detected along with the PCR product. On the other hand, with the K-12 template, only the 152 bp product was observed whereas that obtained with *E. coli* O157:H7 yielded both 152 and 625 bp PCR products (Fig. 1). The specificity of this multiplex PCR was tested against some non-pathogenic, enterotoxigenic, enterohemorrhagic, and enteroaggregative strains of *E. coli*. The PCR-amplified products of 152 and 625 bp

Table 1. Amplification parameters of different PCR programmes used in the study.

S. No.	Name of programmes	Denaturation		Annealing		Extension	
		Temperature	Time	Temperature	Time	Temperature	Time
1	EcUidR	94°C	1 min	59°C	1 min	50°C	1 min
2	EcFliCH7	94°C	15 s	65°C	15 s	72°C	75 s
3	EcUH6250	94°C	30 s	62°C	30 s	50°C	1 min
4	EcUH6272	94°C	30 s	62°C	30 s	72°C	75 s
5	EcUH6365	94°C	30 s	63°C	30 s	65°C	1 min

Fig. 1. Specificity of multiplex PCR for detection of EHEC strains using primers targeted against 'uidR' and 'fliCH7' genes.

Lanes: 1, 100 bp ladder; 2, EPEC-011 ab: NM; 3, EHEC-933; 4, EAggEC; 5, ETEC-309; 6, ETEC-271; 7, ETEC-113; 8, O157:H7 (CMC); 9, O157:H7 (NIZO); 10, NCTC; 11, ATCC; 12, EHEC-933; 13, H10407; 14, K-12; 15, negative control.

could be observed with two of the *E. coli* strains, namely *E. coli* O157:H7 and EHEC-933 only (Fig. 1). All other *E. coli* strains produced the 152 bp band only. Hence, this multiplex PCR assay was used for further studies. Since the multiplex PCR-positive EHEC-933 strain examined in our study was not thoroughly characterized, it is possible that it may belong to serotype *E. coli* O157:H7 itself or to serotype O55:H7, which has also been claimed to be detected by a *fliCH7*-based set of primers (Gannon et al., 1997). These investigators also reported that a 625 bp PCR product could also be detected with several other h7 strains and could only be differentiated by *RsaI* restriction digestion. However, *RsaI* digestion of 625 bp PCR product could not be set-up in the present study due to which the results can not be corroborated with those of Gannon et al. (1997).

This multiplex PCR assay was also tested for its specificity against other pathogens. The PCR amplified products specific for *uidR* (152 bp) and *fliCH7* (625 bp) could not be observed with any of the bacterial strains tested except *Shigella dysenteriae*, where a 152 bp product was obtained (data not shown). However, the *fliCH7* primer set was found to be highly spe-

cific for *E. coli* O157:H7 and did not give any product with other *E. coli*, non-*E. coli* or lactic acid bacteria. Hence, the multiplex PCR assay developed in our study is highly specific for *E. coli* O157:H7.

Sensitivity of the multiplex PCR assay using *uidR*- and *fliCH7*-based primers

The standardized PCR assay was also evaluated for sensitivity by extracting DNA from the LB broth spiked with different levels of cells ranging from 10 to 10⁶ CFU. The PCR-amplified products of 152 and 625 bp were obtained even when template was extracted from 10 CFU, which indicates the high sensitivity of the PCR assay (Fig. 2). Our results in this regard are similar to the observations made by Bej et al. (1991) and Gannon et al. (1997), who also observed high specificity and sensitivity of the PCR assay developed using *uidR* and *fliCH7* sets of primers. Our results do indicate that the multiplex PCR assay developed in our lab was extremely sensitive and specific for detection of *E. coli* O157:H7 from pure cultures of the target organism. Efforts were subsequently made to apply this technique both in spiked as well as naturally contaminated milk

Fig. 2. Sensitivity of multiplex PCR assay in broth and milk samples spiked with *E. coli* O157:H7 cells.

Lanes: 1, 100 bp ladder; 2-7 (broth): 2, 1,000,000 CFU; 3, 1,00,000 CFU; 4, 10,000 CFU; 5, 1,000 CFU; 6, 100 CFU; 7, 10 CFU; 9-13 (milk): 9, 1,00,000 CFU; 10, 10,000 CFU; 11, 1,000 CFU; 12, 100 CFU; 13, 10 CFU; 14, *E. coli* O157:H7 (positive control); 15, negative control.

samples for direct detection of *E. coli* O157:H7 after making appropriate modifications.

Application of multiplex PCR in spiked milk samples

Before applying the multiplex PCR assay using *uidR* and *fliCH7* sets of primers to naturally contaminated milk samples, this assay was first tested on milk samples spiked with the target organism at different levels as described previously. *E. coli* O157:H7 could be detected from milk samples spiked with as low as 10 CFU, as shown in Fig. 2, thereby indicating that the assay was fairly sensitive. However, further sensitivity of this multiplex PCR could not be accomplished since we did not test milk samples with only one cell due to inherent handling errors. Our results in this regard are almost comparable with those of Gooding and Choudary (1997) and Desmarchelier et al. (1998), who were able to detect as low as one CFU of *E. coli* O157:H7 in ice cream and spiked milk samples. However, both these groups had used pre-enrichment step in their respective PCR assays. In comparison to PCR assays developed by those two groups of workers, the total time taken for detection of 10 CFU of *E. coli* O157:H7 from spiked milk samples by our multiplex PCR assay was 6 h without enrichment and less than 10 h after enrichment. The enrichment step definitely improved the efficiency of PCR assay but at the cost of some additional time. Nevertheless, from the results obtained from our multiplex PCR assay and PCR methods developed by other workers, it can be concluded that PCR assays are extremely sensitive to detect even low levels of *E. coli* O157:H7 which the conventional methods based on culturing techniques may not be able to detect. This finding could be of considerable significance since occurrence of even one *E. coli* O157:H7 cell in a food sample could cause health problems on ingestion due to their subsequent multiplication in the gastrointestinal tract or the food itself during storage.

Application of multiplex PCR in naturally contaminated raw milk samples

The results pertaining to detection of *E. coli* O157:H7 in naturally contaminated milk samples by multiplex PCR before and after 4 h enrichment in CT-SMAC broth have been presented in Fig. 3. Out of a total of 24 milk samples examined in this experiment, *E. coli* O157:H7 could be clearly detected from only one of the samples without any enrichment (Fig. 3, lower gel,

Fig. 3. Detection of *E. coli* O157:H7 in market raw milk samples by multiplex PCR.

Upper gel (UG): Lanes: 1, BMVI marker; 2-7 (before enrichment): 13-18; 8-13 (after enrichment): 13-18; 14, *E. coli* O157:H7; 14, negative control. Lower gel (LG): Lanes: 1, BMVI marker; 2-7 (before enrichment): 19-24; 8-13 (after enrichment): 19-24; 14, *E. coli* O157:H7; 15, negative control.

lane 4), as indicated by the appearance of 625 bp (*fliCH7* specific) and 152 bp (*uidR* specific) amplified products. However, when the samples were enriched in CT-SMAC broth, three of the samples (Fig. 3, lower gel, lane 8, 9, 10) clearly demonstrated both the PCR products, indicating the presence of *E. coli* O157:H7 in these samples. The most salient feature of our multiplex PCR assay was that it could work successfully for monitoring even naturally contaminated raw milk samples for *E. coli* O157:H7 without any problem due to PCR inhibitors. In this context, the template DNA extraction method developed in our lab could provide PCR inhibitor-free template DNA from raw milk, and hence could account for clean and reproducible PCR amplification products specific for *E. coli* and *E. coli* O157:H7. The results of this multiplex PCR assay were also validated by microbiological analysis of the milk samples on CT-SMAC/Chrom agar plates which revealed the presence of typical *E. coli* O157:H7 colonies. However, the identification of *E. coli* O157:H7 is tentative as *RsaI* restriction digestion of the PCR amplified product was not carried out in our study. Nevertheless, this finding indicated that the possibility of this high risk pathogen gaining access into milk and milk products produced in India does exist. However, there are not enough reports available to support our results based on PCR based detection of *E. coli* O157:H7 from naturally contaminated milk samples. Most of the information available on these lines is based on detection of this pathogen from samples after spiking with *E. coli* O157:H7 (Desmarchelier et al., 1998; Gooding and Choudary, 1997; Mukhopadhyay and Mukhopad-

hyay, 2007; Paton and Paton, 1998). There are only a handful reports which substantiate our findings concerning PCR-based detection of *E. coli* O157:H7 directly from natural milk samples. In this regard, the results of Meade and Stark (1996) merit special mention as they reported the occurrence of SLT (Shiga-like toxin)-producing *E. coli* in 11% of the tank raw milk samples in Germany. Apart from this, 75% of the samples had also contained the 'eae' gene. In contrast to this, Desmarchelier et al. (1998) did not detect *E. coli* O157:H7 in any of the 147 raw milk samples even after enrichment. Caro et al. (2006) reported that only 3 ewe's milk samples were positive for *rfb*O157 and *fliCH7* genes after 22 h incubation. All *E. coli* O157:H7 isolates were shigatoxigenic, predominantly stx2, and harbored additional genes related to virulence such as *ehxA* and *eaeA*. Rey et al. (2006) reported the incidence of O157:H7 serotype only in one bulk tank of unpasteurized milk. Similarly, Karna et al. (2007) depicted a low incidence of O157:H7 in bulk tank milk, however suggesting that a risk from other enteropathogenic and EHEC forms of *E. coli* may exist.

Application of multiplex PCR in pasteurized milk samples

The efficacy of the multiplex PCR assay developed in our lab was also tested in pasteurized milk samples for the detection of *E. coli* O157:H7. On critical appraisal, none of the 24 samples examined showed the

fliCH7 gene product (625) specific for *E. coli* O157:H7. However, the *uidR*-specific PCR product of 152 bp could be detected invariably from 10 (before enrichment) and 15 (after enrichment) of the pasteurized milk samples (Fig. 4). The signal of the *uidR*-specific PCR product appeared to be weak in most of the positive unincubated pasteurized milk samples, which could be attributed either to availability of low number of target organisms in the samples or ascribed to false positive reaction with non-viable organisms. However, the signal improved considerably after enrichment, perhaps due to multiplication of viable cells in pasteurized milk. The microbiological analysis also revealed that the number of cells increases up to 10^4 CFU/sample after the enrichment step. On a critical note, the detection of *E. coli* in pasteurized milk samples examined in this study does not rule out the possibility of post-pasteurization contamination of the samples with *E. coli*.

From the foregoing results, it could be concluded that the multiplex PCR assay developed in our lab is extremely sensitive, specific and rapid, and hence, could be extremely valuable for detection of even low levels of *E. coli* O157:H7 even in naturally contaminated milk samples.

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Fig. 4. Detection of *E. coli* O157:H7 in pasteurized milk samples by multiplex PCR.

Upper gel (UG): Lanes 1, BMVI marker; 2-7 (before enrichment): 1-6; 8-13 (after enrichment): 1-6; 14, *E. coli* O157:H7; 15, negative control. Lower gel (LG): Lanes 1, BMVI marker; 2-7 (before enrichment): 7-12; 8-13 (after enrichment): 7-12; 14, *E. coli* O157:H7; 15, negative control.

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