Indian Journal of Animal Sciences **82** (2): 204–208, February 2012

Rapid and sensitive detection of *Escherichia coli* in milk by 16S rRNA gene targeted PCR

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Received: 10 September 2010; Accepted: 30 August 2011

ABSTRACT

The present study was conducted to optimize PCR amplification targeted against *Escherichia coli* DNA for detection of 14 different pathogenic and non pathogenic strains of this organism in milk. 16S rRNA gene is present as multiple copy gene, thus targeting this gene enhances the sensitivity of the assay by 1000–5000 times. The template DNA from broth cultures and milk (spiked as well as natural) was extracted using a modified method of Pospiech and Neumann followed by agarose gel electrophoresis of genomic DNA. The PCR parameters were optimized using different combinations of 16S rRNA gene based primers, namely, 16E1, 16E2 and 16E3. The PCR conditions optimized included initial denaturation at 95°C/5 min followed by 35 cycles each of denaturation, at 94° C/30 sec; annealing at 60°C/30 sec and extension at 72°C/30 sec followed by final extension at 72°C/2 min. Using all the primers mentioned above, the PCR amplified a 584 bp product that was specific for *E. coli.* The lowest level of DNA that could be detected by PCR assay was 1 ng and 10 cells of *E. coli* in broth. *E. coli* could be recovered from almost all the raw milk samples and their number ranged from 0.17×10^4 to 8.1×10^4 cfu/ml and 1.7×10^4 to 87×10^4 cfu/ml before and after enrichment, respectively.

Key words: *E. coli*, Milk, PCR, 16S rRNA

The presence of *Escherichia coli* in foods could be of considerable public health significance because it is considered as an index of faecal contamination (Dufour *et al*. 1977) and likely presence of other enteric pathogens in foods. Some *E. coli* serotypes/strains may be pathogenic and directly involved in outbreaks of infantile diarrhoea (Villalobos *et al*. 2008, Ochoa *et al*. 2009) and other serious ailments like hemolytic colitis (Pistone Creydt *et al*. 2005), hemolytic uremic syndrome (Zoja *et al.* 2010) and thrombotic thrombocytopenic purpura (TTP) (Karpac *et al*. 2008).

Denny *et al*. (2008) reported outbreaks of *E. coli* associated with raw milk consumption. The conventional methods for detection of *E. coli* include the culturing on selective media (Greenberg 1985), bioluminescence and hybridization. However, these techniques are extremely laborious, time consuming and hence, are of limited use to the dairy industry. To overcome these problems, PCR was developed for detection of this microorganism in water (Bertrand and Roig 2007) but when this technique is applied to foods, the enzymatic activity of *Taq* DNA polymerase is adversely affected due to infinite arrays of ingredients in milk that include proteins, carbohydrates, fat, oils, chemicals and numerous other compounds. 16S rRNA gene is present as multicopy, thus enhances the sensitivity of the assay by 1000–5000 times. In the light of these considerations the present study was undertaken to optimize PCR amplification targeted against 16S rRNA gene for detection of *E. coli* in broth and milk.

MATERIALS AND METHODS

Bacterial strains and media: The bacterial strains used in the study included pathogenic and non pathogenic *E. coli* strains. The *E. coli* non pathogenic strains included ATCC, DH5α, E-10, NCTC, K-12 while pathogenic strains included EAggEC, EHEC 933, O157:H7, EPEC 011ab:NM, ETEC 271; 309; 113, H1047,ST/LT. All the strains were cultured in LB broth at 37° C for 24 to 48 h.

Extraction of template DNA: The genomic DNA from all the test bacterial cultures was extracted by following the modified method of Pospiech and Neumann (1995). Briefly, the aliquots of 200 µl each of n-propanol, 25% ammonia, petroleum ether and diethyl ether were added to 1 ml of milk samples. The samples were mixed thoroughly and centrifuged at 4,500 rpm for 20 min in a 2 ml safe lock tube. The disc

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Table 1. Oligonucleotide primers used in PCR for the detection of *E. coli*

Gene	Primers	Sequence	Amplified product	Reference
16S rRNA	16 E1	52 GGGAGTAAAGTTAATACCTTTGCTC32	584bp	Tsen *et al.* -1998
	16 E2	52 TTCCCGAAGGCACATTCT32		
	16 E3	52 TTCCCGAAGGCACCAATC32		

and the supernatant were removed. The pellet was resuspended in 50 ml sterilized MQ H_2O and the suspension was transferred to a 1.5 ml Eppendorf tube. The above sample was centrifuged for 5 min/1000 rpm and the supernatant was discarded. The pellet was resuspended in 200 µl of SET buffer containing lysozyme at the rate of 1 mg/ml and incubated at 37° C for 1 h. The above step was followed by addition of 1/ 10^{th} volumes of 10% SDS and 0.5 mg/ml proteinase K and further incubated at 55° C with occasional inversion for 2 h. One third volume of 5 M NaCl and one volume of chloroform were then added to the above mix and incubated at room temperature for 30 min with frequent inversion. The samples were centrifuged at 4,500 rpm for 15 min. The aqueous phase was transferred to the new tube. The DNA was precipitated by adding one volume of ethanol and then washed with 70% ethanol. The DNA pellet was dried under vacuum in a speed Vac System for 10 min and dissolved in 20 µl TE buffer (pH 8.0) containing 10 mg/ml of RNAase A. The extracted genomic DNA preparation along with the tracking dye was used for further experiments.

Oligonucleotide primers: All the oligonucleotide primer pairs i.e. 16S rRNA were custom synthesized. The sequence of these primers, their target genes used in this study are given in Table 1. All the primers were designed based on published information and were tested for temperature using OLIGO version 5.0 programme.

PCR reaction and agarose gel electrophoresis: Before setting up of amplification reaction, all the reagents were thawed except Taq DNA polymerase, mixed and briefly centrifuged. The reaction mix, comprising PCR buffer, dNTPs and primers was prepared generally in advance. All the ingredients except template DNA was added to the mastermix (Taq DNA polymerase, 2.5U; template DNA, 500 ng) and template DNA (5µl i.e. 500ng) was added in the end just before transferring the reaction tubes on to the thermal cycler. The final volume of the PCR reaction was adjusted to 25 µl. For PCR, template DNA was initially denatured at 95°C for 5 min. Next 30–35 cycles were programmed (Tsen *et al.* 1998). Finally an additional extension was given at 72° C for 2 min. PCR products obtained were electrophoresed using a 2.0 percent agarose gel.

Raw milk sample collection: Raw milk samples were collected in 50 ml sterilized Falcon tubes. The collected milk was transported to the lab within 30 min. The milk was processed for DNA isolation as described earlier.

RESULTS AND DISCUSSION

E. coli was targeted to apply PCR for rapid detection of this organism in milk, as in India—this organism is on routine basis to assess the microbiological quality of dairy foods.

An attempt was made to standardize the extraction of template DNA of *E. coli* from broth and milk following the modified method of Pospiech and Neumann (1995). Initially, the PCR reaction conditions as used by Tsen *et al.* (1998) were followed in our study using different combinations of 16E1/16E2/16E3 primers. For each PCR cycle, denatuaration, annealing and extension were carried out at 94°C/20 sec; 56° C or 58°C/20 sec and 72°C/30 sec, respectively. Non specific bands were detected on agarose gel, when annealing temperatures of either 56°C and 58°C were used in this study. However, when the annealing temperature was raised to 60° C, all the non-specific bands disappeared amplifying only the product size of expected size of 584 bp. Hence for further studies, the optimized PCR cycling conditions used in our assay were: initial denaturation 95°C/5 min; 35 cycles each of denaturation 94°C/30 cycles; annealing 60° C/30 sec and extension, 72°C/30 sec followed by final extension at 72°C/2 min.

For the detection specificity of 16S rRNA based primers against *E. coli* strains when PCR assay was performed using 16E1/16E2 or 16E1/16E3, a 584 bp PCR product was

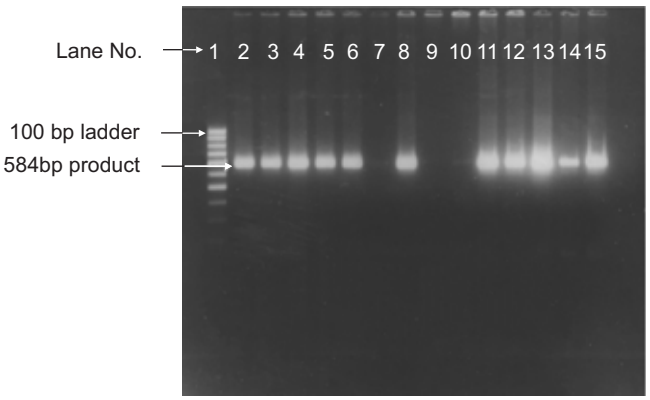

Fig. 1. Amplified PCR products of DNA templates from different *E. coli* brothcultures using all the three primers 16E1, 16E2 and 16E3 simultaneously.

Lanes: 1.100bp ladder; 2. E-10; 3.K-12; 4. DH5a; 5. 0.157 (CMC); 6. 0.157 (NIZO), 7. EPEC 011ab NM; 8. EHEC-933;9.ETEC-309;10. ETCE271; 11. ETEC-113; 12. EAggEC; 13. H10407; 14. ATCC; 15. NCTC

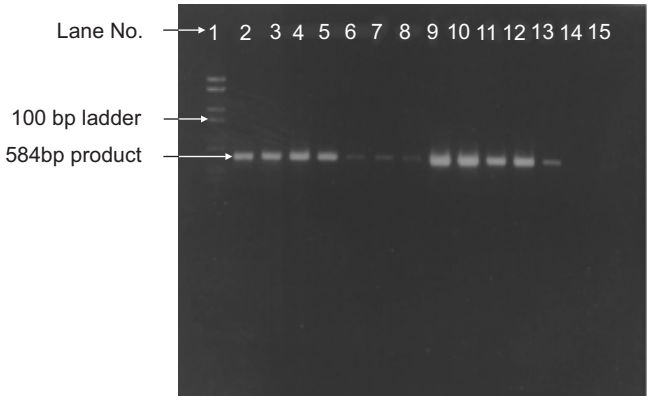

Fig. 2. Sensitivity of PCR assay after amplification with template DNA from *E. coli*-12 and0.157: H7 using primers 16E1, 16E2 and 16E3. Lane l: 100bp ladder; Lanes 2–8 (K-12) : 2. 1000 ng, 3. 500 ng, 4. 100 ng, 5.50 ng, 6. 20 ng 7. 10 ng, 8. 01 ng; Lanes 9–15 (0157H7): 9. 1000 ng, 10. 500 ng, 11. 100 ng, 12. 50 ng, 13. 20 ng, 14.10 ng, 15.01 ng

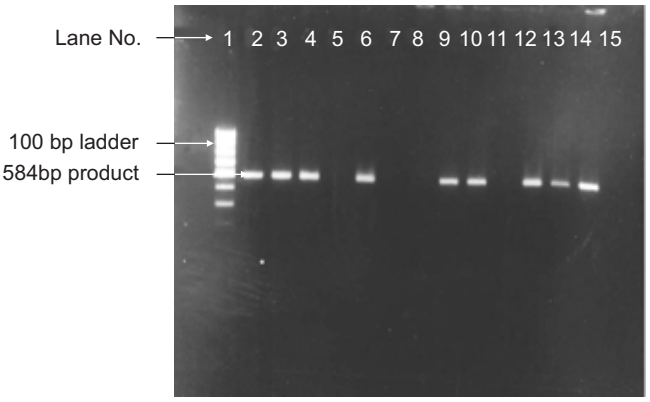

Fig. 3. Sensitivity of PCR assay in broth and milk samples spiked with different levels of cells of *E. coli* K-12 using primers 16E1/16E2/16E3. Lane 1: 100bp ladder; Lanes 2–7 (Broth): 2. 1,000,000; 3.100,000; 4. 10,000; 5. 1000; 6. 100; 7.10; Lanes 8–13: 8. 1,000,000; 9.1,00,000; 10. 10,000; 11.1000; 12. 100; 13.10; Lane 14: K-12, Positive control; Lane 15: Negative control

observed initially in few strains (data not shown). However when all the 3 primers were combined in 1 PCR reaction and tested against different *E. coli* strains, the specific amplified product of 584 bp was detected with all the non pathogenic as well as pathogenic strains of *E. coli* (Fig.1). Our results in this context appear to be consistent with the findings of *Tsen et al.* (1998) who could also consistently get a 584 bp product when all the 3 primers were used in their PCR assay, thereby clearly suggesting some variations in the 16S rRNA regions targeted for designing of the primers.

In order to determine sensitivity of our PCR assay, the broth samples were spiked with different concentrations of template DNA. As can be evidenced from Fig. 2, the specific

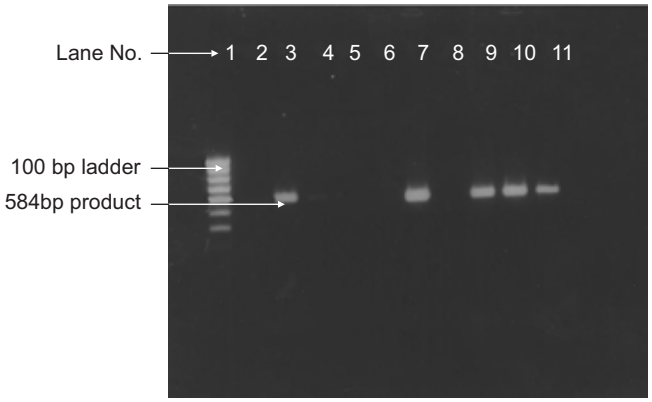

Fig. 4. Detection of *E. coli* in naturally contaminated raw milk samples by 16S rRNA based PCR assay. Lane 1: 1,100bp ladder; Lanes 2–6 (raw milk samples): 2. S1; 3. S2; 4.S3; 5. S4; 6. S5; Lanes 7: K-12 (Positive control), Lane 8. Negative control; Lane 9: E-10; Lane10. ATCC; Lane 1. NCTC

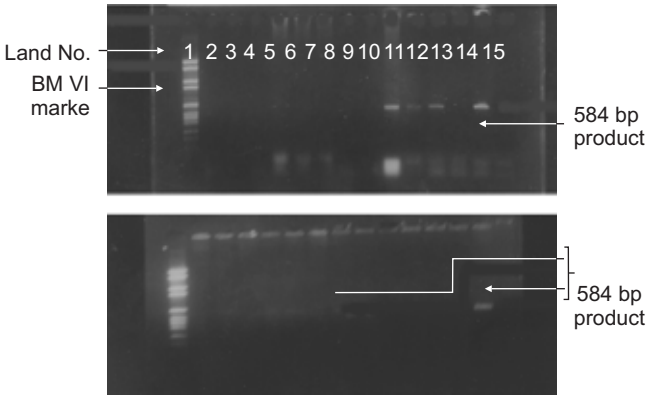

Fig. 5. Detection of *E. coli* by 16S rRNA based PCR assay in naturally contaminated raw milk samples before and after enrichment.

Upper Gel: Lane 1; Lanes 2–7 (without enrichment); Lane 8–13 contaminated raw milk samples; Lanes 14–15

EM VI marker: 2. 1, 3; 3. 2, 4. 3; 5. 4; 6. 5; 7. 6; 8. 1; 9. 2; 10. 3; 11. 4; 12. 5; 13. 6; 14. *E coli* K-12 (positive control) 15. Negative control

Lower Gel: Lane 1; Lane 2–7: Pre-emid for 2 hr; Lane 8–13 (included raw milk samples); Lane 14–15

EM VI Maker: 2. 7; 3. 8; 4. 9; 5. 10; 6. 11; 7. 12; 8. 7; 9. 8; 10. 9; 11. 10; 12. 12; 13. 12; 14. K-12 (positive control) 15. Negative control

amplified 584 bp PCR product could be detected even with 1 ng of template DNA. Similarly, when broth samples were spiked with different levels ranging from 10^6 to 10 cells of *E. coli*, the PCR product could be detected from broth samples seeded with as low as 10 cells of *E. coli* (Fig. 3). However, the signal appeared to be rather weak. Our results in this regard are comparable with those of Mona (1996) who could also detect very low levels of coliforms and *E. coli* from pure broth cultures of *E. coli* in her multiplex PCR assay.

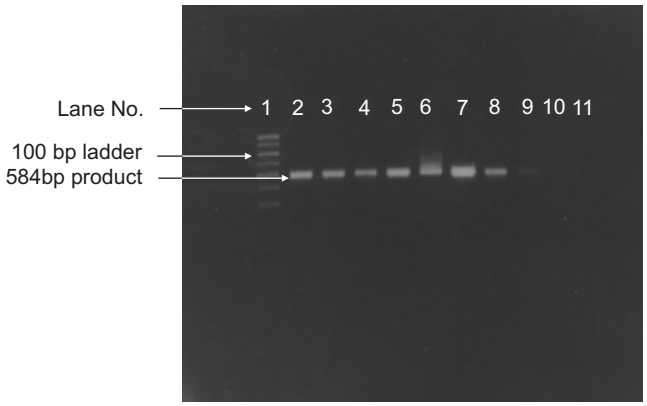

Fig. 6. PCR using 16E1/ 16E2/16E3 from natural samples with additional phenol: chloroformtreatment of template DNA
Lanes: 1, 100bp ladder; 2–4 milk samples (nnincnbated); 5–7, Milk samples (incubated); 8, Milk spiked with K-12; 9, K-12 (Broth); 10, Negative control)

We randomly selected naturally contaminated raw milk samples collected from different sources. The specific 584 base pair PCR product could be detected from 11 raw milk samples (Fig. 4). After carrying out initial testing of our PCR assay on random raw milk samples, we conducted a systematic and comprehensive survey of raw milk sampled from different sources for monitoring the presence of *E. coli* with our PCR assay. The same samples were also subjected for enumeration of *E. coli* by culturing the EMB agar to validate our PCR results. The data pertaining to our PCR results of 24 samples before and after 4–h pre-enrichment based on the comprehensive survey of raw milk samples has been presented in Fig.5 a and b. As can be inferred from Fig.5a, b, a total of 3 raw milk samples without enrichment showed 584 bp *E. coli* specific product on gel. However, around 50% of raw milk samples after 4 h pre-enrichment in Mac Conkey's broth gave positive signal indicating that pre-enrichment improved the performance of our PCR assay considerably. This could be attributed to multiplication of the targeted organisms vis a vis non targeted contaminants to provide reasonably good amount of template DNA for amplification in the PCR assay. Another possible explanation for improvement in the performance of PCR by prior 4-h enrichment of raw milk samples in Mac Conkey's broth is dilution of residual PCR inhibitors as was reported by Lampel *et al.* (1996). Caro *et al.* (2006) investigated the presence of *E. coli* O157:H7 in raw ewe's milk samples, by a multiplex PCR assay for presence of rfbO157 and fliCH7 genes. Of all the ewe's milk samples studied, 3 were positive for *E. coli* O157:H7. They could not get colonies after an enrichment of 6 h incubation. They used an incubation period of 22 h. In this context, our results are certainly better than Caro *et al.* (2006), whereas they used an enrichment of 24 h while we used only 4-h enrichment step thus reducing the detection time.

In order to improve our PCR assay, we introduced an additional phenol-chloroform extraction step in our template DNA extraction for preparation of template DNA before subjecting it to PCR assay. The results pertaining to the effect of additional phenol-chloroform step are presented in Fig. 6. As is clearly discernable from the results, the PCR amplification signals specific for *E. coli* were improved considerably. *E. coli* could be unequivocally detected from both of the raw milk samples (lanes 3 and 4) and 4 of the spiked milk samples (lanes 7, 9, 10 and 11). From these results, it can be concluded that incorporating a 4 h pre-enrichment step and an additional phenol-chloroform treatment during template DNA preparation, enhances the performance of PCR assay significantly.

It may be concluded that our 16S rRNA based PCR assay using a combination of 16E1, 16E2 and 16E3 primers could be an extremely valuable and powerful tool for rapid detection of *E. coli* in milk and other dairy foods in less than 10 h. This method can be easily adopted by dairy industry in India for routine monitoring of dairy foods for *E. coli*. Application of such PCR based detection methods in dairy industry in India will not only enhance the credibility of their products among the Indian consumers but also raise the standards of our dairy in international market.

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