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Research Article

DIARRHOEA CAUSING *E. COLI* IN PASTEURIZED MILK

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ABSTRACT

Seventy five of eighty pasteurized milk samples collected from local market of a city of northern India were positive for blue black colonies having greenish metallic sheen on EMB selective medium presuming them as *E.coli.* However, 54 (67.5%) of them were characterized as *E.coli* biochemically. The segment of *pal* gene, which is conserved in all *E.coli*, was amplified by PCR assay that enhanced the number of *E.coli* positive samples to 64 (85.3%) and highlighted the superiority of the PCR over biochemical identification**.** These 64 *E.coli* isolates were further characterized by PCR assay using gene specific primers and amplification of the *eae* gene of enterohaemorrhagic *E.coli* (EHEC) and LT and ST toxin coding genes of enterotoxigenic *E. coli* (ETEC). Of these, 18 were confirmed as EHEC, 2 as LT toxin producing ETEC and 44 as other types of *E. coli*. PCR technique proved to be a rapid and specific alternative of *E.coli* identification and characterization into EHEC and ETEC. Antimicrobial sensitivity test revealed resistance of *E. coli* isolates to erythromycin (44/64; 68.7%), cephradine (38/64; 59.4%) and ceftazidine (21; 32.8%) and sensitivity to ofloxacin (56/64; 87.5%), ciprofloxacin (58/64; 90.6%) and norfloxacin (62/64; 96.8%).

Key words: *E. coli,* pasteurized milk, PCR, antimicrobial sensitivity

Under the impression of milk being pasteurized is safe, public may consume it without heating but the reports of milk borne illnesses have been correlated with such practices. Hence, health concern is a prime objective for evaluation of quality of pasteurized milk. Reports of identification of human pathogens like *E. coli* O157:H7 (Guodong *et al.,* 1997), *Listeria monocytogenes* (Fleming *et al.,* 1985), *Yersinia enterocolitica* (Ackers *et al.,* 2000). *Salmonella spp.* (Ryan *et al.,* 1987) and *Bacillus cereus* (Langeveld *et al.*, 1996) from pasteurized milk are not uncommon. Pasteurized milk contaminated with *E. coli* has been the main cause of several outbreaks of milk borne diseases since 1980s and thus remains a serious health risk (Allmann *et al.,* 1995). *E. coli* 0157:H7 which causes haemorrhagic colitis and haemolytic uremic syndrome has been responsible for several outbreaks associated with consumption of improperly processed pasteurized milk (Wang *et al.,* 1997). In recent years, *E. coli* has been recognized as a serious food-borne pathogen and has been associated with numerous outbreaks in UK, Japan and USA (Scotter *et al.,* 2000).

Pasteurized milk sold in the market worldwide is considered safe for drinking without boiling and carries labeled shelf life of two to three weeks. However, there are dairy companies in India that sell pasteurized milk with shelf life warrantee of only two or three days. The safety of pasteurized milk available in the market is not known and hence necessitated this investigation. The present study was undertaken to detect diarrhoea causing enterohaemorrhagic (EHEC) and enterotoxigenic *E. coli* (ETEC) in the pasteurized milk available in local market.

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MATERIALS AND METHODS

Collection of Samples: Eighty samples of pasteurized milk belonging to four different brands, viz. A, B, C and D (20 samples of each brand) were collected aseptically from poly-packs available in local market. The process of sample collection extended over a summer period of five months from May to September, 2010.

Plating on EMB: 0.1 ml each of undiluted milk and its 1:10 dilution were plated on eosin methylene blue (EMB) agar by surface spreading technique and the plates were incubated at 37ºC for 24 h. Colonies having blue black colour with greenish metallic sheen were considered as presumptive *E. coli.* These colonies were purified by growing them thrice in BHI broth and streaking on EMB. The purified culture was stored in maintenance media till their identification and characterization.

Identification and Characterization of the Presumptive Isolates of *E. coli*: The isolates were subjected to biochemical tests, polymerase chain reaction

(PCR) assay and antimicrobial sensitivity test. Presumptive isolates of *E. coli* were identified as per the methods described by Edwards and Ewing (1972) and MacFaddin (1976).

Molecular Identification of *E. coli* Isolates: All the presumptive isolates were characterized by PCR using the specific primers for *E. coli* and their further categorization into EHEC and ETEC. DNA was extracted from the *E. coli* isolates and known positive cultures of *E. coli* using ZR Fungal/Bacterial DNA kit (Zymo Research Corp., USA) following the recommended extraction procedure by the manufacturer.

PCR assay was done on the extracted DNA as per the procedure described by Parekh and Subhash (2008). The primers specific to *pal* gene of *E. coli* (Kuhnert *et al.,* 1995), *eae* gene of EHEC (Ellingson *et al.,* 2005) and ST and LT toxin-encoding genes of ETEC (Galbadage *et al.,* 2009) were employed to identify presumptive *E. coli* and its characterization into EHEC and ETEC (Table 1). Initially, the extracted DNA of each presumptive *E. coli* was confirmed as *E. coli* using *pal* gene and then the *E. coli* isolates were subjected to PCR assays using primers for *eae* gene of EHEC and ST and LT toxin-encoding genes of ETEC. Amplified segments of *pal* gene of all *E. coli* as 280 bp, *eae* gene of EHEC as 360bp, LT toxin encoding genes of ETEC as 322 bp and ST toxin encoding gene of ETEC as 147 bp PCR products identified *E. coli*, EHEC, LT toxin producing ETEC and ST producing ETEC, respectively.

Antimicrobial Sensitivity Testing: *E. coli* isolates were subjected to 14 antimicrobial drugs by disc diffusion technique (Bauer *et al.,* 1966). The antibiotic discs employed were cephalosporins (cefoxitin, 30 mcg; cephradine, 25 mcg; ceftazidine, 30 mcg; cefixime, 5 mcg; ceftriaxone, 30 mcg; cefuroxime, 30 mcg), quinolone (ofloxacin, 5 mcg), flouroquinolones (nalidixic acid, 30 mcg; ciprofloxacin, 5 mcg; norfloxacin,10 mcg), tetracycline (doxycycline, 30 mcg), aminoglycoside (gentamicin, 10 mcg), nitrofuran (nitrofurantoin, 30 mcg) andmacrolides (erythromycin, 15 mcg). The results were interpreted according to the manufacturer's (HiMedia) recommendations.

Table 1

Primers specific to *E. coli*, EHEC and ETEC used in this study

Type of *E. coli*	Primer	Primer Sequence
E. coli	ECPAL-L	5'GGCAATTGCGGCATGTTCTTCC3'
	ECPAL-R	5'CCGCGTGACCTTCTACGGTGAC3'
EHEC	EHEC-F	5'TGGTACGGGTAATGAAAA3'
	EHEC-R	5'AATAGCCTGGTAGTCTTGT3'
ETEC LT	LT-F	5'GAGTACTTCGATAGAGGAACTCA3'
toxin	LT-R	5'GATCCGGTGGGAAACCTGCT3'
ETEC ST	ST-F	5'TGGATGCCATGTCCGGAGGT-3'
toxin	ST-R	5'CAACAGGTACATACGTTACAGAC3'

RESULTS AND DISCUSSION

Isolation and Biochemical Characterization: Out of 80 milk samples, 75 (93.75%) were found to have blue black colonies with characteristic greenish metallic sheen on EMB agar. The purified cultures of such colonies were considered as presumptive *E. coli*. Being Gram's negative, catalase positive and oxidase negative,indole and methyl red positive, VP, citrate and urease negative, acid production from lactose, glucose and sorbitol, variable reactions of acid from sucrose, lysine, ONPG, nitrate and glucuronidase positive, the isolates were biochemically characterized as *E. coli*. Eighteen isolates showed a negative reaction to sorbitol. Fifty four (72%) of 75 presumptive isolates were therefore, characterized biochemically as *E. coli*. The remaining 21(28%) presumptive isolates were having one or more variable biochemical reactions and therefore considered biochemically non-*E. coli*.

PCR Assay: A band of 280 bp was observed on gel using PCR product of known *E. coli* (Fig. 1). All the 75 presumptive isolates from pasteurized milk were similarly subjected to PCR. Amplified PCR products of 64 (85.33%) of these 75 isolates produced the electrophoretic band of 280 bp (Fig. 1) identifying them as *E. coli*. No band was observed in the remaining 11 isolates indicating them to be non-*E. coli.*

Fifty four (72%) of the 75 presumptive isolates could be identified as *E. coli* biochemically while 64 (85.3%) could be identified as *E. coli* by PCR using primers from *pal* gene, a conserved gene in all types of *E. coli* (Kuhnert *et al.,* 1995). The PCR identified all 54 biochemically confirmed isolates as *E. coli* and 10 more isolates as *E. coli* that gave variable biochemical reactions. This may be due to variability in one or more biochemical tests which have made them biochemically negative but genetically positive. Based on USA and European regulations, pasteurized milk must be *E. coli* negative (Hillerton and Berry, 2004; McLaughlin, 2006). The results of *E. coli* in milk samples of this study are worrisome because *E. coli* in pasteurized milk may cause severe diarrhoea in newborn and adolescents. *E. coli* in pasteurized milk may be due

to post-pasteurization contamination or due to unhygienic procedures of processing, handling, delivering and selling of milk (Anderson *et al.,* 1995). In recent years, *E. coli* has been recognized as a serious food-borne pathogen and has been associated with numerous outbreaks of disease in UK, Japan and USA (Scotter *et al.,* 2000). Different workers have reported the presence of *E. coli* in pasteurized milk (Allmann *et al.,* 1995; da Silva *et al.,* 2001; Santoro *et al.,* 2007).

All the 64 PCR positive *E. coli* were subjected again to PCR using primer set specific assays to *eae* gene of EHEC. A band of 360 bp was observed in 18 (28.12%) isolates (Fig. 2) confirming them to be EHEC.

When remaining 46 non-EHEC isolates (71.88%) were subjected to PCR to identify ST toxin producing ETEC and LT producing ETEC, only 2 (3.13%) isolates were identified as LT toxin producing ETEC as their amplified PCR products produced electrophoretic bands of 322 bp (Fig. 3). No specific band at positions 322 bp was observed in the remaining 44 *E. coli* isolates suggesting them to be non-ETEC. None of the 46 isolates was positive for ST as no band of 147 bp was observed.

While comparing results of biochemical characterization and molecular characterization of 75 presumptive isolates of *E. coli*, 54 (72%) were confirmed as *E.coli* biochemically while this number increased to 64 (85.33%) by PCR. Eighteen (28.12%) and two (3.13%) of these 64 isolates were confirmed as EHEC and LT toxin producing ETEC, respectively. All the isolates confirmed negative by PCR showed variable biochemical reactions while all the biochemically positive isolates were identified as *E. coli* by PCR. Again all the isolates confirmed to be EHEC and ETEC by PCR were preliminarily confirmed as *E. coli* by biochemical tests. Hence the two tests complemented each other.

Five types of diarrhoea producing *E. coli* are known (Vernam and Evans,1991). Pasteurized milk is the heated milk at defined time and temperature combination and is supposed to be free of pathogens. Presence of diarrhoea producing *E. coli* in such milk reflects a faulty pasteurization process. Though heating/boiling the pasteurized milk before consumption is a practice in Indian households, yet the practice of consuming pasteurized dairy products without heating by many groups of people in India is also not uncommon. Presence of EHEC and ETEC in pasteurized milk available in the market is therefore alarming and possibility of milk borne outbreaks cannot be ruled out. Pre-treatment contamination, inadequate pasteurization and post-pasteurization contamination are the likely factors for such outbreaks. In this study, two of the 64 *E. coli* were characterized as LT toxin producing

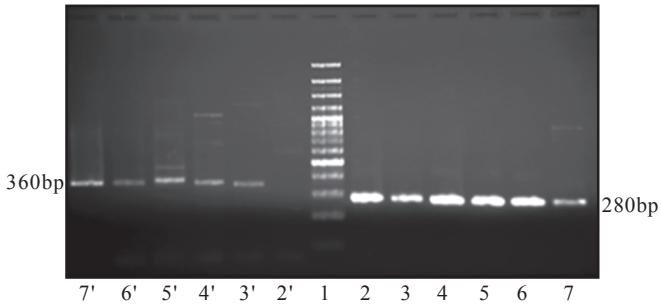

Fig 2. Agarose gel electrophoresis of PCR products showing *E. coli* and EHEC. Lane 1=100 bp ladder as molecular marker; Lanes 2 and 3=*E. coli* positive controls; Lanes 4-7=*E. coli* positive isolates; Lanes 2'=EHEC negative isolate; Lanes 3'-5'=EHEC positive isolates; Lanes 6'-7'=EHEC positive controls

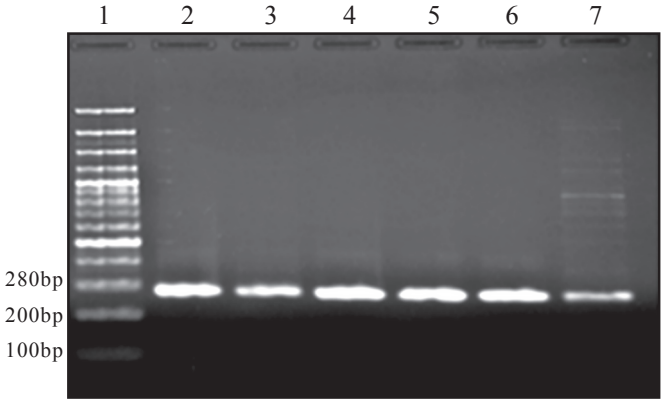

Fig 1. Agarose gel electrophoresis of PCR products using *pal* gene primers. Lane 1: 100bp ladder as molecular marker; Lanes 2 and 3= *E. coli* positive controls; Lanes 4-7= *E. coli* field isolates

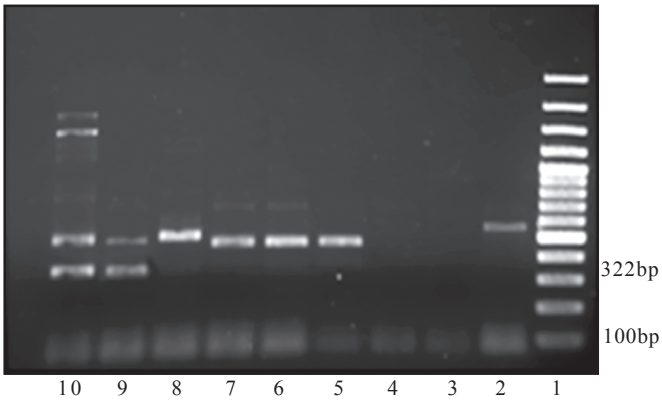

Fig 3. Agarose gel electrophoresis of PCR products showing ETEC positive isolates. Lane 1=100 bp ladder as molecular marker; Lanes 2-8 (ETEC negative isolates), Lanes 9-10 (ETEC positive isolates).

Table 2
Antibiogram of *E. coli* isolates (n= 64)

Antimicrobial class	Antimicrobial agent	No. of isolates sensitive	No. of isolates intermediary sensitive	No. of isolates resistant
Cephalosporins	Cefoxitin, 30 mcg; cephradine, 25 mcg; ceftazidine, 30 mcg; cefixime, 5 mcg; ceftriaxone, 30 mcg; cefuroxime, 30 mcg	13 to 54 (20.31- 84.38%)	7 to 13 (10.94-20.32%)	2 to 38 (3.12- 59.40%)
Fluoroquinolones	Nalidixic acid, 30 mcg, Ciprofloxacin, 5mcg; Norfloxacin,10 mcg	36 to58 (56.25-90.63%)	06 to18 (9.38 -28.13%)	0 to10 (15.63%)
Quinolone	Ofloxacin, 5mcg	62 (96.88%)	02 (3.12%)	0
Tetracycline	Doxycycline, 30 mcg	50 (78.13%)	07 (10.94%)	07 (10.94%)
Aminoglycoside	Gentamicin, 10 mcg	48 (75.0%)	10 (15.63%)	06 (9.38%)
Nitrofuran	Nitrofurantoin ,30 mcg	49 (76.56%)	07 (10.94%)	08 (12.50%)
Macrolide	Erythromycin, 15 mcg	01 (1.56%)	19 (29.69%)	44 (68.75%)

ETEC which is an alarming situation. These LT toxin producing strains produce severe symptoms and closely resemble those of *Vibrio cholerae* 01, both in type and severity (Jay, 1978). Hamada *et al.* (2000) have reported the outbreaks of heat-stable enterotoxin producing *E. coli* in Japan.

Antibiogram of 64 isolates of *E. coli* to 14 antimicrobial drugs is given in Table 2. A highest number of isolates were resistant to the erythromycin (68.75%) and cephradine (59.40%), while the resistance to ciprofloxacin, ofloxacin, ceftriaxone and norfloxacin (0%-3.12%) was lowest. Ofloxacin, ciprofloxacin and norfloxacin were the drugs found most sensitive as 62 (96.88%), 58 (90.63%) and 56 (87.5%) isolates showed sensitivity, respectively.

Fourteen antimicrobial agents comprising six belonging to cephalosporins, three to fluoroquinolones and one each to tetracycline, quinolone, aminoglycoside, nitrofuran and macrolide were used to study. The selection of these agents was made due to their common use in medical practice to treat enteric infections. Maximum sensitivity of *E. coli* isolates was observed against quinolone and fluoroquinolone whereas the lowest sensitivity was against macrolide. Similar results had been reported by Dubey *et al.* (2001) and Kumari *et al.* (2002). Among the six antimicrobials belonging to cephalosporin group, the maximum sensitivity of *E. coli* strains (54/64, 84.3%) was to ceftriaxone while strains were least sensitive to cephradine (13/64, 20.3%). From the results of this study, it can be interpreted that quinolones and fluoroquinolones are more effective against *E. coli* from pasteurized milk than second generation cephalosporins. However, further study is required by testing a larger number of strains of *E. coli* from pasteurized milk.

The study reveals the presence of *E. coli* as well as enterohaemorrhagic and enterotoxigenic strains of *E. coli* in pasteurized milk sold in market. The presence of ETEC and EHEC strains in milk assumes significance from public health point of view. *In-vitro* antimicrobial sensitivity test revealed varied resistance of *E. coli* strains to different antimicrobials.

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