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. 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 O157: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.

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 37°C 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 MacFadden (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 360 bp, 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; cefazidine, 30 mcg; cefixime, 5 mcg; ceftriaxone, 30 mcg; cefuroxime, 30 mcg), quinolone (ofloxacin, 5 mcg), fluoroquinolones (nalidixic acid, 30 mcg; ciprofloxacin, 5 mcg; norfloxacin, 10 mcg), tetracycline (doxycycline, 30 mcg), aminoglycoside (gentamicin, 10 mcg), nitrofurantoin (nitrofurantoin, 30 mcg) and macrolides (erythromycin, 15 mcg). The results were interpreted according to the manufacturer’s (HiMedia) recommendations.

**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 diarrhea 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

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**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 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 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).
ETEC which is an alarming situation. These LT toxin producing strains produce severe symptoms and closely resemble those of *Vibrio cholerae* O1, 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 norflaxacin (0%-3.12%) was lowest. Ofloxacin, ciprofloxacin and norflaxacin were the drugs found most sensitive to 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.

### REFERENCES


