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Detection of pathogenic strains of *E. coli* in milk products sold in and around Chandigarh

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Abstract: *Milk and milk based product are an integral part of the culinary habits of people all over India but they are highly susceptible to contamination by a variety of microorganism. Among all micro-organisms Escherichia coli is a frequently contaminating organism. Some virulent strains of E.coli such as Shiga toxin-producing Escherichia coli (STEC) are responsible for hemorrhagic colitis and hemolytic uremic syndrome in humans. The present study was carried out to detect the presence of pathogenic strains of E. coli capable of producing shiga toxin2(stx2) in milk products (Rasgulla, gulabjamun, milk-cake) sold at various sweet shops in and around Chandigarh. Total 316 samples of milk products were analyzed and E.coli was detected in 12 samples. After PCR screening two isolates of E. coli were found to be stx2 positive . From the results of the present study it can thus be concluded that shiga toxin producing strains of E. coli, which are resistant to a number of antibiotics, are present in some of the milk products sold in Chandigarh. Even though the number of positive samples is small, more strict preventive measures should be followed during the preparation of milk products keeping in mind the health of the consumers.*

Key words: *E. coli*, milk products, pathogenic strains, Chandigarh

Introduction

Milk is a major part of human food and plays a prominent role in our diet. About 45% of milk produced is consumed as fluid milk. About 35% is processed into butter or ghee; about 7% into Paneer (cottage cheese) and, about 4% is converted into milk powder; and the rest is used for production of other products such as Dahi (yoghurt) and sweet meats (Kumar et al.,2011). It has been reported that milk products like Khoa, Gulabjamun, Rasgulla and Rasmalai get easily contaminated by a variety of microorganism because of their high nutritive value and complex chemical composition (Grewal & Tiwari,1990; Soomro et al., 2003; Kumar & Prasad, 2010).

Escherichia coli is mostly used in the microbiological analysis of food as an indicator of poor hygiene conditions (Chatterjee et al.,2006). Several strain of enteropathogenic *E. coli* have been found to be associated with outbreaks of gastroenteritis and food poisoning in human beings (Singh & Ranganathan,1978). Shiga-like toxin producing *Escherichia coli* has emerged as an important food-borne pathogen. The organism has been found to be responsible for several food-borne outbreaks (Ostroff et al.,1990).

Conventional methods used for detection for *E. coli* include enrichment and isolation using media such as E. coli (EC) broth, lauryl sulfate tryptose 4-methylumbelliferyl- b-D-glucuronic acid broth, eosin methylene blue agar, and MacConkey sorbitol agar (Vanderzant & Spittstoesser, 1992). All of these media, however, are not capable of identifying STEC strains. However, molecular approaches can be designed to be specific (Fode-Vaughan et al.,2003). Toma *et al.*(2003) used multiplex PCR assay for the identification of human diarrheagenic *Escherichia coli*. Mansouri-Najand *et al.*(2007) also analyzed the prevalence of *E. coli* O157 and non-O157 STEC in raw milk cheeses produced in the Southern part of Iran by using multiplex-PCR method.

Several studies have been carried out to detect presence of *E.coli* in milk and milk products sold under market conditions at different places. Soomro *et al.* (2002) isolated *Escherichia coli* from raw milk and milk products sold at Tandojam, Pakistan. Out of 160 samples 88 samples were found to be positive for *E.coli*. Similarly Oksuz *et al.* (2003) reported *E. coli* 0157:H7 at the rate of 1% in 100 samples of raw milk. *E. coli* has also been isolated from milk products like Mawa, Khoa, Cream, Dahi, Cheese, Butter, gulabjamun (Kumar & Sinha, 1989; Kulshrestha, 1990). Pedha and burfi have also been found to be contaminated with *Escherichia coli, Salmonella chottmueleri, Shigella flexeneri, haemolytic Streptococci* and *Pseudomonas aeruginosa* (Patel, 1984).

Singh & Prakash (2008) analyzed 116 samples of cottage cheese and curd to detect the presence of *E. coli, Staphylococcus aureus* and *Listeria cytogenes*. Out of the 116 bacterial isolates obtained from cottage cheese, 15 were confirmed as *E. coli* whereas in case of curd samples out of 58 bacterial isolates, 5 were confirmed as *E. coli*. It has been reported that immune-compromised

individuals are more prone to food-borne infection (Altekruse et al., 1994) and these infection in HIV positive individuals can be life threatening (Bhatnagar et al., 2007).

Shiga toxigenic *E.coli* have been isolated from a number of food products in India like chicken meat (Zende *et al.*, 2013),fish and fish products (Kumar *et al*., 2009; Surendraraj *et al*., 2010; Gupta *et al*., 2013) but to the best of our knowledge no study has been carried out to detect these pathogenic strains in milk products sold in Chandigarh. Keeping in view the above facts, the present study was designed to detect the presence of pathogenic shiga toxin producing strains of *E. coli* in milk products (Rasgulla, gulabjamun, milk-cake) sold at various sweet shops in and around Chandigarh.

Materials and Methods

Collection of samples: 316 samples of milk products (107 samples of Rasgullas, 108 samples of Gulabjamun and 101 samples Milk-cake each) were collected in sterilized glass bottles from different region of Chandigarh city and were brought to the laboratory under low temperature. Samples were stored at 4^{o}C and processed within 24 hours.

Detection of *E.coli* : A portion from the centre of each sample of milk product was extracted aseptically, inoculated in 100 ml sterile enrichment broth (lactose broth), and then incubated at 37^{o}C for 24 hrs. All the samples positive for *E. coli* were identified by spreading the 100µl of the inoculum from the lactose broth tubes on the Eosin Methylene Blue Agar, incubated at 37˚C for 24 hrs. The plates were observed for the growth of *E. coli*. *E.coli* form colonies with green metallic sheen. A single colony was picked and sub cultured on Eosin Methylene Blue Agar for the purification of the isolate.

Identification, morphological and biochemical characterization of isolated *E. coli* strains:

The bacterial colonies isolated after purification were initially Gram stained and the isolates were biochemically characterized and confirmed to be *E. coli* by performing catalase, Indole, methyl red, Voges-Proskauer, Simmon's citrate and Triple Sugar Iron agar or TSI test (Table 1).

Table 1: Biochemical Characterization of *E.coli*

S.No	BIOCHEMICAL TEST	REACTION
1	Catalase test	+ ve
2	Indole production test	+ve
3	Methyl-red	+ve
4	Voges- Proskauer test	-ve
5	Simmon's citarate test	-ve
6	Triple Sugar Iron agar test	A/A+gas

DNA isolation of the characterized *E.coli* strains

Isolation of genomic DNA was done by chloroform- phenol method of Naravaneni & Jamil (2005).

Characterization of Shiga Toxigenic *Escherichia coli* By PCR Assay for *stx2* gene

To investigate the virulence potential of *E. coli*, isolates were subjected to Polymerase chain reaction (PCR) as described by Gupta *et al*. (2013). PCR primer pairs for *stx2* gene were designed according to sequence data published by Paton & Paton (1998). Reference strain used in this study was shiga toxin producing *E.coli* strain (MTCC 433), which was obtained from IMTECH, Chandigarh. Details of the sequence of primer used are given in Table 2.

Table 2: Sequence of PCR Primers for *stx2* gene

Primer	Sequence(5'-3')
*stx2*F	GGCACTGTCTGAAACTGCTCC
*stx2*R	TCGCCAGTTATCTGACATTCTG

The reaction mixture for PCR was prepared in 25µl volume; containing 2.5µl 10X PCR buffer,1.5 Mm $MgCl_2$,0.2mM dNTP mix,12.5pmol/µl of each forward and reverse primers (Bioserve, India),1 unit of Taq DNA Polymearse and required quantity of purified DNA template (1-5µl). Amplification was done in a thermocycler (Master cycler Gradient, Eppendorf, Germany) under the following conditions: each cycle consist of 1 min of denaturation at 95°C, 2min of annealing at 65°C for 1st 10 cycles; decrementing 1°C in each cycle by 11th cycle to 15th cycle and from 15th cycle to 35th at 60°C and 1.30 min of elongation at 72°C for 1st25 cycles and incrementing 6 seconds in each cycle from 26th cycle to35th cycle followed by final extension at 72°C for 5 min. *E. coli* reference strain (MTCC 433) was included as a positive control. After PCR, products were subjected to agarose gel electrophoresis and were visualized under UV trans-illuminator.

Determination of Antibiotic sensitivity of pathogenic *E.coli* strains

Antibiotic sensitivity of pathogenic strains of *E.coli* was done by Bauer's disc diffusion method (1966). The antibiotics used were Ampicillin, Amikacin, Tetracyclin, Cefotaxime, PenicillinG, Gentamycin, Erythromycin, Cefixime and Chloramphenicol. All antibiotic disks used were of Himedia (Hi media Laborotories, Mumbai, Ltd.) brand. The concentration of antibiotics used were Cefixime (30 µg), Chloramphenicol (5 µg), Ampicillin (25 µg), Tetracycline (20 µg), Cefotaxime (30 µg), Penicillin-G (10 units),

Gentamicin (120 µg), Erythromycin (15 µg), (E^{15}), Amikacin (30 µg), (AK^{30}).

The cultures were inoculated into sterile nutrient broth and enriched by overnight incubation at 37°C. Enriched cultures were then swabbed over sterile Mueller Hinton agar plates using sterile cotton swabs. After 15 minutes of pre-diffusion time, the antibiotic discs were dispensed over the seeded agar plates which were then incubated at 37°C for 16–18 hours and the diameter of the inhibitions zones were measured. The results were then compared with the interpretative chart supplied by the Hi-Media laboratories and strains were classified as sensitive, intermediate and resistant.

RESULTS

In the present study, 316 samples of milk products were analyzed for the presence of pathogenic strains of *E.coli*. Out of 316 samples, 12 samples were found to be contaminated with *E.coli*. The highest contamination was recorded in the samples of Rasgulla, which showed 5(4.6%) samples out of 107 positive for *E.coli* followed by Milk-cake (3.95%) and Gulabjamun (2.7%) samples (Table 3).

Table 3: Overall percentage of *E.coli* contamination in milk product samples

Milk product	No of samples screened	No of samples positive for *E.coli*	Percentage contamination
Rasgullas	107	5	4.67%
Gulabjamun	108	4	3.70%
Milk-cake	101	3	2.97%
Total	316	12	3.79%

To assess the virulence potential of the *E.coli* isolates, Polymerase chain reaction (PCR) was carried out for well known virulence gene *stx2*. DNA was isolated from confirmed *E.coli* strains using Chloroform- phenol method (Fig 1). PCR screening of isolates revealed that 2 (16.66%) out of 12 *E.coli* isolates were positive for *stx2* (Fig 2).These pathogenic strains were designated as M8 and R47.

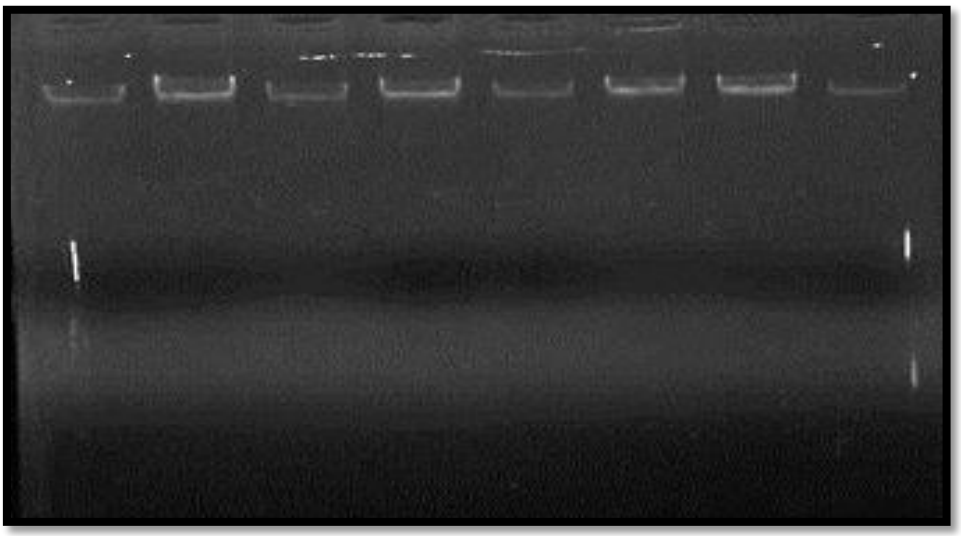

Fig1: Agarose gel electrophoresis showing the bands of isolated genomic DNA of *E.coli*

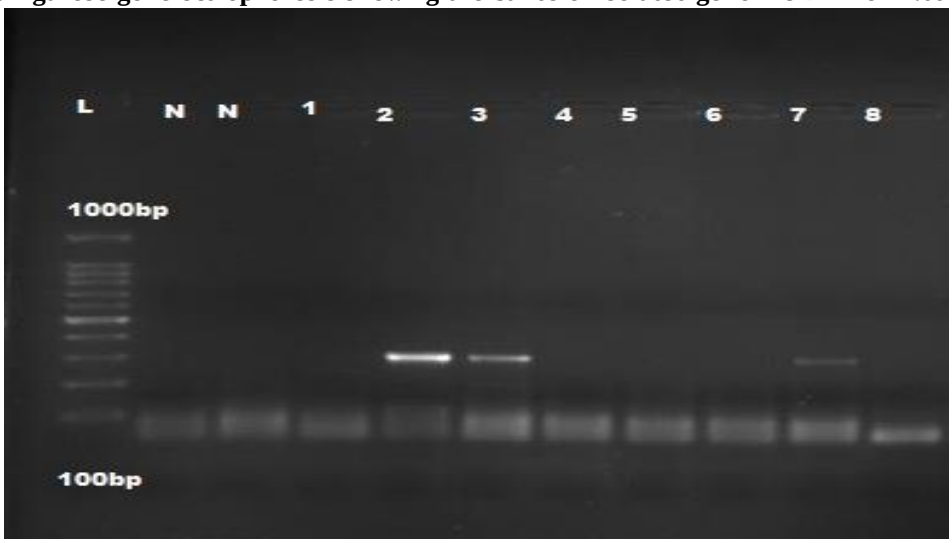

Fig2: Agarose gel showing amplification of *E.coli stx2* gene product; N: negative control, L: DNA ladder, lane no. 2, 3 contain positive sample of *E.coli* & 7 contain indicator strain (MTCC 433).

When antibiotic sensitivity pattern of these pathogenic *E.coli* isolates was determined, strains M8 and R47 showed resistance to a number of antibiotic, namely against Ampicillin, Cefotaxime, Penicillin, Erythromycin. The isolates were found to be sensitive to Chloramphenicol and Gentamycin. The indicator strain was found to be resistant against Penicillin and sensitive to Chloramphenicol, Tetracycline, Gentamycin, Amikacin and Cefixime (Table 4).

Table 4: In Vitro Antimicrobial drug sensitivity of pathogenic *E.coli* isolates

Strain name	Name of Antibiotic								
	Chloramphenicol	Ampicillin	Tetracycline	Cefotaxime	Penicillin G	Gentamycin	Erythromycin	Amikacin	Cefixime
M8	S	R	S	R	R	S	R	I	S
R47	S	R	I	R	R	S	R	I	I
MTCC433	S	I	S	I	R	S	I	S	S

S = susceptible to the given antibiotic, R= Resistant to the given antibiotic, **I** = Intermediate resistance

DISCUSSION

From the literature review of various studies carried out in India to detect the presence of *E.coli* in milk products, it can thus be concluded that most of theses milk product samples are contaminated with *E.coli*, the degree of contamination though vary from one place to another. According to Kumar & Prasad (2010) main reason for contamination of these products is that their methods of manufacturing, handling, sale and transportation are traditional. Handling of sweets with bare hands, not using hair covering and handling of money during serving may also contribute to contamination of milk products. According to Microbial Food Safety Regulation of India, *E. coli* must be absent in 1g sample of Khoya/ Chhana/ Paneer as per the Prevention of Food adulteration rules, 1956.

In the present study *E.coli* was detected in only 3.79% of the milk products, indicating that better hygienic conditions are maintained during their manufacturing at Chandigarh as compared to other places in India. Maity et al. (2010) reported that 27.91% of the milk products sold in Kolkata were contaminated with *E.coli*. Similarly *E.coli* was detected in 29.09% of the milk products sold in Jalandhar city (Kumar et al., 2011).

Though in the present study the numbers of contaminated samples were less but if these strains are pathogenic then consumption of these products may cause food-borne illness. The foods reported to be most commonly involved in food-borne disease are meat and meat products, poultry, eggs, milk and milk products, sweetmeats and rice preparations (Tambekar & Bhutada, 2004). Several strains of enteropathogenic *E.coli* have been found to be associated with outbreaks of gastroenteritis and food poisoning in human beings (Singh & Ranganathan, 1978).

STEC (shiga toxigenic *E.coli*) may belong to a very broad range of O serogroup , with in the human disease associated strains though those which are *stx2* positive appears to produce serious complications like HUS (haemolytic uremic syndrome) (Nataro *et al.*,1998). PCR screening of isolates in the present study revealed that 16.66% of *E.coli* isolates were positive for *stx2* and these strains were found to be resistant to a number of antibiotics, namely against Ampicillin, Cefotaxime, Penicillin, Erythromycin. Resistance to ampicillin has also been observed by Schroeder et al. (2002) in *E. coli* strains isolated from humans, cattle, swine, and food. Another study (Rahimi et al., 2011), in Iran reported that the *E. coli* strains isolated from traditional cheese, ice cream, and yoghurt were resistant to ampicillin and erythromycin.

From the results of the present study it can thus be concluded that milk products, available in Chandigarh are contaminated with antibiotic resistant toxic strains of *E. coli*. Considering the public health importance of consumers, it is needless to say that more strict preventive measures should be followed and the products should be prepared hygienically so as to reduce the bacterial load present in it. Milk should also be boiled or pasteurized before consumption and preparation of different milk products.

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