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ORIGINAL RESEARCH

Study on isolation, molecular detection of virulence gene and antibiotic sensitivity pattern of *Escherichia coli* isolated from milk and milk products

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Abstract

Aim: The study was undertaken to isolate pathogenic *E. coli* from milk and various milk products, detection of virulence gene using Polymerase chain reaction (PCR) and investigate their antibiotic sensitivity pattern.

Materials and Methods: Altogether 250 milk and various milk products samples consisting of raw milk (50), cheese (50), ice-cream (50), mawa (50) and dahi (50) were collected from milk vendors, retail shops located in Anand city, under aseptic precautions. For the enrichment of the organism from the collected samples, MacConkey broth was used and inoculation was carried out on MacConkey agar and EMB agar. Later on, to confirm the isolates, various biochemical tests such as IMViC test, Urease test were performed. Evaluation of antibiotic sensitivity pattern of *E. coli* was assessed by disk diffusion method. Finally the *E. coli* isolates were screened for the presence of virulence associated genes by PCR .

Results: The prevalence of *E. coli* was observed 32 % in the samples comprising of milk (52.00%), cheese (28.00%), ice-cream (20.00%), mawa (44.00%), and dahi (16.00%). Antibiotic sensitivity was recorded high for Co-trimoxazole (100%) followed by Gentamicin (96.73%), Trimithoprime (93.47%) and Doxycycline hydochloride (92.39%). Least sensitivity was recorded for Ampicillin (8.69%). In this study, out of 80 *E. coli* isolates, 25 isolates (31.25%) were positive for *stx* genes, of which 7 (8.75%) isolates were positive for *stx*1 gene only, while 12 (15.00%) isolates were positive for *stx*2 gene only and 5 (6.25%) isolates were positive for both *stx*1 and *stx*2, 7 isolates (8.75%) were positive for *eaeA* gene and all the isolate were negetive for *rfb* O157 gene.

Conclusions: Current study supports the finding that raw milk and various milk products can be regarded as critical source of pathogenic *E. coli* This explains the need of strict monitoring and surveillance for effective measures of hygiene and sanitary practice during production of milk and various milk products.

Keywords: antibiotic sensitivity test, enteropathogenic *E. coli,* milk, milk products, PCR

Introduction

Raw milk consumers have existed in various parts of the world. World milk production reached 724 million tons in 2010 as per Food and Agriculture Organization, resulting in trade and massive consumption of various milk products [1]. Raw milk is consumed directly by a large population in rural areas. Milk is an excellent medium for the growth of numerous microbes which produce consequential spoilage of the milk and various milk products or infections in consumers [2]. Because of the specific production, it is impossible to avoid contamination of milk with microorganisms therefore the microbial content of milk is a major feature in determining its quality [3]. The existence of food borne pathogens in raw milk may increases the threat of ingestion and transmission of food borne pathogens and ingestion of harmful toxins [4]. Huge numbers of microbes can get access to milk and various milk products including *E. coli* which is an indicator of fecal contamination, constituting a public health hazard.

The most important causes of food borne diseases are Shiga toxin-producing *E. coli* (STEC) among the other seropathotypes of *E. coli* [5]. STEC produce various complications including diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) [6]. Report indicate that consumption of raw milk and various milk products related with occurrence of 1 to5 per cent of food infections and among that 53 per cent of cases produced by enteropathogenic *E. coli* (EPEC) [7].

In view of the these particulars, the current study was undertaken to detect and characterize the *E. coli* from milk and various milk products, collected from the milk vendors, retail shops in and around Anand city, Gujarat.

Materials and Methods

Sample collection: Altogether 250 milk and various milk products samples consisting of raw milk (50), cheese (50), ice-cream (50), mawa (50) and dahi (50) were collected from milk vendors, retail shops located in Anand city, under aseptic precautions. The milk samples were collected in sterilized sample bottle and other milk products sample were collected in sterilized polyethylene bags in morning hours were transported to the P. G. research laboratory of the Veterinary Public

Table-1. Details of primers used for PCR.

Target Genes	Primer sequence (5'→3')	Product Size (bp)	References
stx1	F:CAGTTAATGTGGTGGCGAAG R:CTGCTAATAGTTCTGCGCATC	894	[11]
stx2	F:CTTCGGTATCCTATTCCCGG R:GGATGCATCTCTGGTCATTG	478	[11]
eaeA	F:GACCCGGCACAAGCATAAGC R:CCACCTGCAGCAACAAGAGG	384	[12]
rfbO157	F:AAGATTGCGCTGAAGCCTTTG R:CATTGGCATCGTGTGGAC	497	[13]

Table-2. *In vitro* antimicrobial drug sensitivity of *E. coli* isolates from milk and milk products

Sr. No.	Antimicrobial agents	Sensitive	Intermediate	Resistant
1	Ampicillin	7(8.75%)	38(47.50%)	35(43.75%)
2	Kanamycin	51(63.75%)	20(25.00%)	9(11.25%)
3	Amikacin	56(70.00%)	12(15.00%)	12(15.00%)
4	Chloramphenicol	64(80.00%)	3(3.75%)	13(16.25%)
5	Oxytetracycline	52(65.00%)	8(10.00%)	20(25.00%)
6	Streptomycin	35(43.75%)	26(32.50%)	19(23.75%)
7	Gentamicin	77(96.25%)	3(3.75%)	0(0.00%)
8	Doxycycline hydrochloride	73(91.25%)	2(2.50%)	5(6.25%)
9	Ciprofloxacin	63(78.75%)	8(10.00%)	9(11.25%)
10	Trimithoprime	74(92.50%)	2(2.50%)	4(5.00%)
11	Levofloxacin	68(85.00%)	4(5.00%)	8(10.00%)
12	Cefotaxime	70(87.50%)	5(6.25%)	5(6.25%)
13	Nalidixic Acid	60(75.00%)	1(1.25%)	19(23.75%)
14	Co-trimoxazole	80(100%)	0(0.00%)	0(0.00%)

Health department in an ice box for further processing and microbiological analysis.

Isolation and identification: Samples were processed to isolate the *E. coli* as per the standard *Bacteriological Analytical Manual* (BAM), U.S. Food and Drug Administration (USFDA) method [8]. The samples were enrichmed in MacConkey broth, the loopful of culture inoculates MacConkey agar. Pink colour colonies obtain from MacConkey agar were taken and inoculate on Eosin methelene blue agar. Greenish metallic sheen colonies obtain on EMB agar were regard as an *E. coli*. Various biochemical tests such as catalase test, Indole production, Methyl red, Voges proskauer, Simon's citrate agar, Urease production, Nitrate reduction etc. were done for the confirmation of *E. coli* as proposed by Edward and Ewing [9].

Antibiotic sensitivity test: The antibiotic susceptibility tests were performed as per method described by Bauer *et al.* [10] to find out the antibiotic sensitivity of *E. coli*. *In vitro* antibiotic sensitivity test of the *E. coli* isolates was conducted by paper disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India). Antibiotics used in this test *viz*. Kanamycin (30µg), Ampicillin (10µg), Streptomycin (10µg), Amikacin (30µg), Cefotaxime (30µg), Oxytetracycline (30µg), Trimithoprime (5µg), Doxycycline Hydochloride (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Nalidixic Acid (30µg), Gentamicin (10µg), Levofloxacin 5µg) and Co-trimoxazole (20µg).

DNA isolation: The DNA of *E. coli* isolates was prepared by using boiling method. First 100 µl of sterilized DNAse and RNAse free water was taken in micro centrifuge tube and approximately loopful of culture was added. Then denaturation was carried out at 95ºC for 10 min, centrifugation was done for the removal of cell debris and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

Polymerase chain reaction (PCR): All the *E. coli* isolates were first screened for the presence of virulence associated genes by using the PCR technique for the detection of different genes. The PCR was standardized for the detection *stx1*, *stx2, eaeA* and *rfb* O157 following the methodology as described by Paneto et al. [11] for detection of *stx1* & *stx2 genes,* El-Jakee et al. [12] for detection of *eaeA* gene and Dhanashee and Mallaya [13] for detection of *rfbO157* with suitable modifications (Table-1). Standardization of PCR was done by using standard strain of *E. coli* O157:H7 and EPEC. The reactions were performed in the thermal cycler (Applied Biosystem, Sweden) with pre-heated lid (Lid temp. 105 °C). For the confirmation of targeted PCR amplification, consisting of 1 µl of 6X gel loading buffer along with 5 µl of the PCR product, then electrophoresis was performed with use of DNA molecular weight marker (Gene Ruler, MBI Fermentas). Agarose gel (2%) along with ethidium bromide (at the rate of 0.5 µg/ml) was used. Electrophoresis was performed in 0.5X TBE buffer at 5V/cm for 60 min. Visualization of amplified product was done under ultraviolet light and was documented by gel documentation system (SynGene, Gene Genius BioImaging System, UK).

Results

Prevalence of *E. coli*: The prevalence of *E. coli* was observed 32% cent in the samples comprising of milk (52.00%), cheese (28.00%), ice-cream (20.00%), mawa (44.00%), and dahi (16.00%).

Prevalence of antibiotic sensitivity of *E. coli*: In present investigation among 80 *E. coli* isolates from milk and various milk products the highest sensitivity was

Table-3. PCR amplification of virulence genes

Sr. No.	Virulence Genes	E. coli isolates positive from				
		Milk	Cheese	Ice cream	Mawa	Dahi
1	*stx1*	2 (8.00%)	4(16.00%)	3(12.00%)	2(8.00%)	1 (4.00%)
2	*stx2*	4 (16.00%)	5(20.00%)	4(16.00%)	5(20.00%)	0(0.00%)
3	*eaeA*	1 (4.00%)	2(8.00%)	2(8.00%)	1 (4.00%)	1 (4.00%)
4	*rfbO157*	0 (0.00%)	0(0.00%)	0(0.00%)	0(0.00%)	0(0.00%)

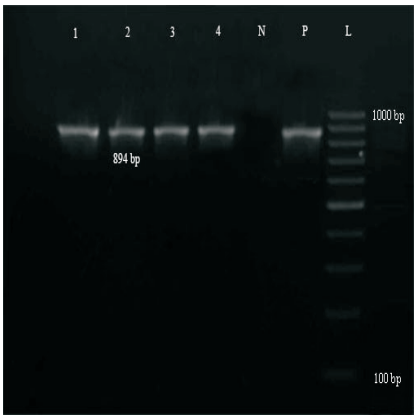

Figure-1. Agarose gel showing PCR amplification of *E. coli stx1* gene product (894 bp) P: Positive control, N: Negative control, L: DNA Ladder, Lane 1 to 4: Positive samples

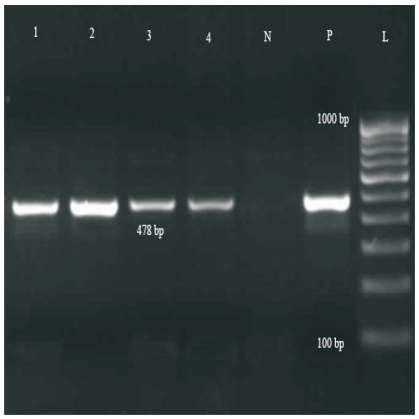

Figure-2. Agarose gel showing PCR amplification of *E. coli stx2* gene product (478 bp) P: Positive control, N: Negative control, L: DNA Ladder, Lane 1 to 4: Positive samples

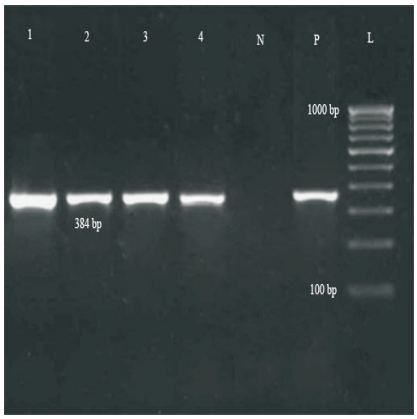

Figure-3. Agarose gel showing PCR amplification of *E. coli eaeA* gene product (384 bp) P: Positive control, N: Negative control, L: DNA Ladder, Lane 1 to 4: Positive samples

recorded for Co-trimoxazole (100%) and least for Ampicillin (8.75%) (Table-2).

The highest resistance was observed against the Ampicillin (43.75%) and least resistance was observed against Doxycycline Hydochloride (6.25%), Cefotaxime (6.25%), Trimithoprime (5.00%) and no resistance was observed against Gentamicin, Co-trimoxazole (Table-2).

Detection of virulence genes: In this study, out of 80 *E. coli* isolates, 25 isolates (31.25%) were positive for *stx* genes, of which 7 (8.75%) isolates were positive for *stx*1 gene only, while 12 (15.00%) isolates were positive for *stx*2 gene only and 5 (6.25%) isolates were positive for both *stx*1 and *stx*2. 12 (15.00%) isolates carried *stx*1 genes and 18 (22.50%) isolates had *stx*2 gene. In this study, 25 isolates from raw milk and various milk products found STEC (Table-3).

Discussion

Prevalence of *E. coli:* In present study prevalence of *E. coli* in milk is 52 per cent, almost similar result was found (57%) by Soomro et al. [14]. However, lower prevalence of *E. coli* than present study was reported 26.43 per cent by Bandyopadhyay et al. [15], 30.28 per cent by Farzan et al. [16], 31.6 per cent by Nanu et al. [17] and 33.96 per cent by Mohd et al. [18]. Prevalence of *E. coli* reported 28 per cent in cheese, the similar result 29.2 per cent in Ras cheese was recorded by Fadel et al. [19]. Higher prevalence 96 per cent in cheese was reported by Paneto et al. (2007). However, lower prevalence of *E. coli* (12.9%) in cottage cheese was reported by Singh and Prakash [20] and 16.6 per cent in cheese was reported by Farzan et al. [16]. Prevalence of ice-cream was found 20 per cent in present study; similar result of prevalence of *E. coli in* ice-cream was reported 16.6 per cent by Farzan et al. [16]. Fadel et al. [19] found that 31.8 per cent of ice-cream samples were positive for *E. coli.* In contrast with this study, high prevalence of *E. coli* (58%) was recorded by Amany et al. [21].

Prevalence of antibiotic sensitivity of *E. coli*: In present investigation among 80 *E. coli* isolates from milk and various milk products the highest sensitivity was recorded for Co-trimoxazole (100%) and least sensitivity was recorded for Ampicillin (8.75%).

The highest resistance was observed against the Ampicillin (43.75%) and least resistance was observed against Doxycycline hydochloride (6.25%), Cefotaxime (6.25%), Trimithoprime (5.00%) and no resistance was observed against Gentamicin, Co-trimoxazole.

Gentamicin, Kanamycin, Streptomycin and Amikacin are commonly used aminoglycosides in the treatment of various microbial infection. In the present study, 23.91 per cent of isolates were reported resistant to the Streptomycin. The similar result was recorded by Mohd et al. [18]. In contrast with present study high resistance was reported 57.89 per cent by Thaker et al. [22]. Ebrahim et al. [23] found that all *E. coli* O157 isolates were susceptible to Streptomycin.

All the *E. coli* isolates were found to be sensitive to Co-trimoxazole in the present study. But least reistance were recorded by Sabry and Elmalt [24], Onono et al. [25], Thaker et al. [22]. Mohd et al. [18] reported 60 per cent sensitivity to co-trimoxazole.

In the present study, 6.52 per cent of *E. coli* isolates from raw milk and various milk products were resistant to Cefotaxime, in contrast with present finding higher resistance was recorded by Mahami et

al. [26] and Mohd et al. [18].

Studies showed that the antibiotic resistance bacteria which exist in the milk of infected animals can be transmitted to human by the ingestion of raw milk or milk products such as cheese, ice cream, mawa, and dahi.

Detection of virulence genes: In this study, out of 80 *E. coli* isolates, 25 isolates (31.25%) were positive for *stx* genes, similar results for lower per cent of *stx* gene positive isolates were reported by Kalliopi et al. [27], Mansouri-Najand and Khalili [28], Caro et al. [29], Stephan et al. [30], Islam et al. [31], Ebrahim et al. [23], Farzan et al. [16], Mohd et al. [18]. On the other hand, high per cent of *stx* positive isolates were reported by Martin and Beutin [32] and Njage et al. [33].

In the present study 12 (15.00%) *E. coli* isolates found positive for *stx*1gene and 18 (22.50%) *E. coli* isolates found positive for *stx*2 gene. Similar finding of predominance of *stx*2 producing strains were reported by Sabry and Elmalt [24], Rey et al. [34], Bandyopadhyay et al. [15]. In contrast with present study predominance of *stx*1 producing strains were reported by Lih Ching et al. [35], Caro et al. [29], Martin and Beutin [32], Njage et al. [33], Farzan et al. [16], Kalliopi et al. [27], Mohd et al. [18].

Out of 80 *E. coli* isolates from raw milk and various milk products, 7 isolates (8.75%) were positive for *eaeA* gene. Similar findings were reported by Lih Ching et al. [35], Lorusso et al. [36], Adjehi et al. [37], Bandyopadhyay et al. [15], Mohd et al. [18]. In contrast to present study high prevalence has also been recorded by Karns et al. [38] and Njage et al. [33]. In some study, not any isolates were found positive for *eaeA* [23,29,30].

In this study, none of the isolate was positive for *rfb* O157 gene. The similar results were reported by Lih Ching et al. [35], Caro et al. [29], and Stephan et al. [30]. In contrast to present study, Mansouri-Najand and Khalili [28] reported one *E. coli* O157 from cheese samples.

Conclusion

The majority of raw milk and various milk products samples were found to be contaminated or carried *E. coli* infections, which require strict management for effective measures for hygiene and sanitary practice. The high percentage resistance in bacterial isolates to ampicilin, oxytetracycline, streptomycin and nalidixic acid requires further research. Continuous efforts are required to reduce the resistance burden in human by strict monitoring of antibiotic resistance of *E. coli from* milk and various milk products samples. PCR based molecular epidemiological studies are required for detection of all types of pathogenic as well as zoonotic potential strains of *E. coli* isolates for future research.

Authors' contributions

PKV, JBN and MNB conceived and designed the study. PKV perfomed the whole study. PKV drafted and revised the manuscript with the help of JBN and MNB. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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