Prevalence and Drug Resistance Pattern of Escherichia coli Strains Isolated from Milk and Milk Products

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Abstract

In the present study prevalence and antibiotic susceptibility pattern of Escherichia coli isolated from milk and milk products from retail dairies of different parts of Udaipur city, Rajasthan were determined. The phenotypic and genotypic characterization of E. coli isolates was done to determine its prevalence and antibiotic susceptibility pattern. A total of 150 samples comprising of raw pooled milk (n=30), pasteurized milk (n=30), dahi (n=30), paneer (n=30), and sweets (burfi) (n=30) were processed for the isolation of E. coli. Out of 150 samples, the prevalence of E. coli was recorded in raw pooled milk, dahi, paneer, pasteurised milk and sweets (burfi) samples as 76.66% (23), 33.33% (10), 20% (6), 0% (0) and 43.33% (13), respectively. The analysis of antibiogram revealed that the most effective antibiotic was Chloramphenicol (91.30%), followed by Trimethoprim to which 86.95% of the isolates were sensitive. Also, 82.60% isolates were sensitive to Gentamicin and Ciprofloxacin, 78.26% to Ceftriaxone, 73.91% to Co-Trimoxazole and other antibiotics were still less effective. Penicillin-G showed highest resistance (100.00%) followed by Methicillin (91.30%), while 52.17% isolates were resistant to Ampicillin, 43.47% to Erythromycin and Carbenicillin, 21.73% to Tetracycline, 17.39% to Ceftriaxone and other antibiotics were still less resistant. Out of 50 E. coli isolates from milk and milk products, only 11 isolates (22%) were found to be positive for blacTTM gene while only 2 isolates (1.25%) were found to be positive for stxl gene. These results indicate that the milk and milk products sold in the study area have high level of antibiotic resistant E coli which is a public health concern. Therefore, stringent hygienic measures and prudent use of antibiotics should be practiced to improve the present worrisome situation.

Keywords

Escherichia coli, Antibiotic resistance, Virulence, Prevalence, Milk

Introduction

Milk is an extraordinarily nutritious food for bacterial growth, which not only spoils the milk and milk products but also can cause infections in consumers [1]. Raw milk contains many microorganisms, because it is regarded as perfect media for microbial
growth [2]. Milk is highly prone to contamination and can serve as an efficient vehicle for human transmission of food borne pathogens, especially Gram-negative bacteria, as these are widely distributed in the environment [3]. Raw milk consumption by humans may be attributed to the lack of knowledge about the food borne pathogens in raw milk. Food borne pathogens are a major threat to food safety, especially in developing countries where hygiene and sanitation facilities are often poor. *Escherichia coli* is among the major cause of outbreaks of food borne diseases [4]. The majority of human infections occur due to the intake of contaminated raw milk products and unpasteurized milk which have been implicated in food borne outbreaks and in sporadic cases of human illness [5].

*E coli* is a parasite living in human and animal intestine. It is among many pathogenic microorganisms which can enter into milk and milk products and is considered as a reliable indicator of contamination by manure, soil, and contaminated water [6,7]. Moreover, different food borne pathogens in milk may be introduced in milk due to the faecal contamination during milking process [8]. Consumption of hygienic foods causes more than 300 diseases worldwide [9,10]. Food borne diseases lead to around 80 million sicknesses, 330,000 hospitalizations, and 6000 deaths in the United States annually [11,12]. Therefore, consumption of raw milk may be linked with the incidence of food-poisoning outbreaks [13]. Also, the emergence of multi drug resistant (MDR) isolates worldwide, pose an additional threat to human health [14].

Presence of *E. coli* in milk and milk products indicates the presence of enteropathogenic microorganisms which is a public health hazard [15]. Different *E. coli* pathotypes are responsible for causing intestinal and extra intestinal infections [16]. Ruminants are the main reservoir and the most significant source of access of STEC in the food chain [17]. Shiga (vero) toxin (Stx)-producing *E. coli* (STEC) is a part of a virulent group of *E. coli* known as enterohemorrhagic *E. coli* (EHEC) [18, 19]. In humans, EHEC cause infections ranging from mild diarrhoea to life-threatening problems, like hemorrhagic colitis and haemolytic uremic syndrome [20,21]. EHEC is linked with bloody diarrhoea and haemolytic uremic syndrome and expresses one or two Shiga-like toxin-encoding genes stx1 and stx2 [22]. Among all *E. coli* pathotypes, ETEC strains cause a cholera-like diarrhoeal disease and are the most common cause of childhood and travellers’ diarrhoea in developing countries [23]. EIEC shows pathogenic phenotypic and genetic similarities with *Shigella spp.* and are associated with dysentery [24].

**Materials and Methods**

A total of 150 samples of milk and milk products were collected (Table 1) from Udaipur city. The samples were processed as per the standard microbiological techniques [25]. The isolation was done by selective enrichment in broth and plating on MacConkey agar (HiMedia). The lactose fermenting colonies were selected and streaked on EMB agar (HiMedia). The colonies producing metallic sheen were selected for further biochemical tests viz., indole test, methyl red test, Voges-Proskauer test, citrate test (IMViC test), TSI test and urease test.

**Serogrouping**

The *E. coli* isolates recovered from milk and milk product samples were serotyped at the National *Salmonella* and *Escherichia Centre* (NSEC), Central Research Institute (CRI), Kasauli, H. P., India.
Polymerase chain reaction for the detection of stx\textsubscript{1} and bla\textsubscript{CTX-M} genes

The primers used in the study are listed in Table 2 and 3. The template DNA was prepared as per the method of HiMedia TM Bacterial Genomic DNA Purification Kit.

The PCR procedure to screen the stx\textsubscript{1} and bla\textsubscript{CTX-M} genes were standardized as described by Hazarika et al., 2007 and Edelstein et al., 2003 with certain modifications. Followed by preliminary trials, the reaction mixtures were optimized to contain 12.5 µl 2 X PCR master mix (Fermentas), 10 pmol of each forward and reverse primer, 7.5 µl nuclease free distilled water and 3 µl of DNA template. The reactions were performed in the thermal cycler (Cole-Parmer) with pre-heated lid (lid temp.=105°C). The cycling conditions were comprised of an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 min and final extension at 72°C for 5 min.

The amplified products were analysed by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 g/ml) along with 100 bp molecular weight DNA marker in horizontal electrophoresis unit (Tarsons). The gel was visualized under UV transilluminator (N&M).

Antimicrobial susceptibility testing of E. coli isolates

All the Escherichia coli isolates were subjected to antibiotic sensitivity test as described by Bauer et al., 1966 [28]. Antimicrobial susceptibility testing was done by agar disc diffusion method. A total of 12 antibiotic discs comprising of Ciprofloxacin, Ampicillin, Co-trimoxazole, Penicillin, Trimethoprim, Carbenicillin, Erythromycin, Chloramphenicol, Tetracycline, Methicillin, Ceftriaxone and Gentamicin were placed on two agar plates each containing 6 antibiotic discs. The zone of inhibition was recorded to determine the susceptibility pattern of the isolates.

Results and Discussion

Prevalence of E. coli in milk and milk products

All the isolates which produced bright pink colonies on MacConkey agar (Fig 1) and colonies with a characteristic metallic sheen on EMB agar (Fig. 2) were selected. Further, the suspected isolates which were found positive for indole and methyl red test while negative for citrate and Voges Proskauer test were confirms as E. coli. Out of 150 samples, the prevalence of E. coli was recorded in raw pooled milk, dahi, paneer, pasteurized milk and sweets (burfi) samples as 76.66% (23), 33.33% (10), 20% (6), 0% (0) and 43.33% (13), respectively.

Serogroups of E. coli isolates

Out of the 23 isolates, 14 isolates of E. coli were typed for ‘O’ antigen. The 14 isolates which could be typed were distributed into 9 different serogroups, whereas 2 isolates did not reacted with the available O group sera (untypable) and 7 were found to be rough. The detailed results of E. coli serogroups of each category are shown in Table 4.

Detection of stx\textsubscript{1} and bla\textsubscript{CTX-M} gene of E. coli

Screening of samples for the presence of stx\textsubscript{1} and bla\textsubscript{CTX-M} gene was done by PCR (Fig 3 & 4). Out of 40 E. coli isolates recovered from milk and milk products, only 2 isolates (1.25%) were found to be positive for stx\textsubscript{1} gene. While, out of 50 E. coli isolates from milk and milk products, only 11 isolates (22%) were found to be positive for bla\textsubscript{CTX-M}
gene. The prevalence of *E. coli* harbouring *stx*<sub>1</sub> and *bla*<sub>CTX-M</sub> gene from milk and milk products is presented in Table 5.

**Table 1** Different types samples collected for *E. coli* isolation

| S. No. | Type of Sample                  | No. of Samples |
|--------|---------------------------------|----------------|
| 1.     | Raw Pooled Milk Samples         | n =30          |
| 2.     | Pasteurized Milk Samples        | n =30          |
| 3.     | Dahi Samples                    | n =30          |
| 4.     | Paneer Samples                  | n =30          |
| 5.     | Sweets (Burfi) Samples          | n =30          |
| Total  |                                | n =150         |

**Table 2** The primers used for the detection of *stx*<sub>1</sub> gene (Hazarika et al., 2007) [26]

| S. No. | Oligo Name | Sequence (5’->3’) | T (°C) | GC-Content | Size of amplified product (bp) |
|--------|------------|-------------------|--------|------------|-------------------------------|
| 1.     | Stx1 F     | CTGCTAATAGTTCTGCGAC | 57.3   | 50 %       | 894 bp                        |
| 2.     | Stx1 R     | CAGTTAATGTGGTGGCGAG | 56.7   | 52.6 %     |                               |

**Table 3** The primers used for detection of *bla*<sub>CTX-M</sub> gene (Edelstein et al., 2003) [27]

| S. No. | Oligo Name | Sequence (5’->3’) | T (°C) | GC-Content | Size of amplified product (bp) |
|--------|------------|-------------------|--------|------------|-------------------------------|
| 1.     | *bla*<sub>CTX-M</sub> F | CGATATCGTGGTGTTGCATCATA | 60.3   | 50 %       | 544 bp                        |
| 2.     | *bla*<sub>CTX-M</sub> R | TTTGCGATGTGCGATTACGTACGATA | 58.9   | 43.5 %     |                               |

**Table 4** The distribution of *E. coli* serogroups milk and milk products

| S. No. | Type of Samples      | Total No. of Samples | No. of Positive Isolates | Prevalence | Serogroups                   |
|--------|----------------------|----------------------|--------------------------|------------|------------------------------|
| 1.     | Raw Pooled Milk Samples | 30                   | 23                       | 76.66 %    | O15, O83, O8, O118, O4, O15, O7, O17 |
| 2.     | Pasteurized Milk Samples | 30                   | 0                        | Nil        | -                            |
| 3.     | Dahi Samples         | 30                   | 10                       | 33.33 %    | -                            |
| 4.     | Paneer Samples       | 30                   | 6                        | 20 %       | -                            |
| 5.     | Sweets (Burfi)       | 30                   | 13                       | 43.33 %    | -                            |
| Total  |                      | 150                  | 52                       | 34.66 %    | -                            |
Table 5: Prevalence of *E. coli* harbouring *stx*<sub>1</sub> and *bla<sub>CTX-M</sub>* gene from milk and milk products

| Name of the gene       | Positive isolate                        |
|------------------------|-----------------------------------------|
| *stx*<sub>1</sub>      | 2 (isolate no. 64, 84)                  |
| *bla<sub>CTX-M</sub>   | 11 (isolate no. 67, 139, 27, 33, 11, 104, 87, 62, 5, 128,9) |

Table 6: Antibiotic resistance profile of the *E. coli* isolates from milk and milk products

| S. N | Antibiotic   | Raw Pooled Milk Samples | Dahi Samples | Paneer Samples | Sweets (Burfi) |
|------|--------------|--------------------------|--------------|----------------|----------------|
|      |              | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) |
| 1    | Penicillin   | 0 (0) | 0 (0) | 100 (23) | 23.07 (3) | 0 (0) | 76.92 (10) | 0 (0) | 0 (0) | 100 (6) | 0 (0) | 0 (0) | 100 (10) |
| 2    | Gentamicin   | 82.60 (19) | 13.04 (3) | 4.34 (1) | 100 (13) | 0 (0) | 0 (0) | 100 (6) | 0 (0) | 0 (0) | 100 (10) | 0 (0) | 0 (0) |
| 3    | Ciprofloxacin | 82.60 (19) | 8.69 (2) | 8.69 (2) | 76.92 (10) | 23.07 (3) | 0 (0) | 100 (6) | 0 (0) | 0 (0) | 100 (10) | 0 (0) | 0 (0) |
| 4    | Trimethoprim | 86.95 (20) | 0 (0) | 13.04 (3) | 100 (13) | 0 (0) | 0 (0) | 83.33 (5) | 0 (0) | 16.66 (1) | 80 (8) | 0 (0) | 20 (2) |
| 5    | Carbenicillin | 30.43 (7) | 26.08 (6) | 43.47 (10) | 15.38 (2) | 15.38 (2) | 39.13 (9) | 16.66 (1) | 33.33 (2) | 50 (3) | 30 (3) | 10 (1) | 60 (6) |
| 6    | Ampicillin   | 34.78 (8) | 13.04 (3) | 52.17 (12) | 30.76 (4) | 76.92 (10) | 61.53 (8) | 0 (0) | 0 (0) | 100 (6) | 10 (1) | 0 (0) | 90 (9) |
| 7    | Erythromycin | 0 (0) | 56.52 (13) | 43.47 (10) | 7.69 (1) | 53.84 (7) | 38.46 (5) | 16.66 (1) | 50 (3) | 33.33 (2) | 0 (0) | 30 (3) | 70 (7) |
| 8    | Chloramphenicol | 91.3 (21) | 0 (0) | 8.69 (2) | 100 (13) | 0 (0) | 0 (0) | 83.33 (5) | 16.66 (1) | 0 (0) | 90 (9) | 10 (1) | 0 (0) |
| 9    | Tetracycline | 17.39 (4) | 60.86 (14) | 21.73 (5) | 76.92 (10) | 53.84 (7) | 38.46 (5) | 33.33 (2) | 33.33 (2) | 33.33 (2) | 0 (0) | 70 (7) | 30 (3) |
| 10   | Methicillin  | 8.69 (2) | 0 (0) | 91.3 (21) | 0 (0) | 0 (0) | 100 (13) | 0 (0) | 0 (0) | 100 (6) | 0 (0) | 0 (0) | 100 (10) |
| 11   | Ceftriaxone  | 78.26 (18) | 4.34 (1) | 17.39 (4) | 84.61 (11) | 7.69 (1) | 7.69 (1) | 50 (3) | 33.33 (2) | 16.66 (1) | 70 (7) | 10 (1) | 20 (2) |
| 12   | Co-Trimoxazole | 73.91 (17) | 13.04 (3) | 13.04 (3) | 100 (13) | 0 (0) | 0 (0) | 83.33 (5) | 0 (0) | 16.66 (1) | 80 (8) | 0 (0) | 20 (2) |

Fig.1 Growth on MacConkey Agar Plates
**Fig. 2** Growth on EMB Agar Plates

**Fig. 3** Agarose gel showing PCR amplified product (894 bp) for \( stx_1 \) gene in *E. coli* isolates
N – Negative control, L – 100 bp DNA Ladder, 64, 84 – Positive Sample

**Fig. 4** Agarose gel showing PCR amplified product (544 bp) for \( bla_{CTX-M} \) gene in *E. coli* isolates
L – 100 bp DNA Ladder, Positive Samples (33, 27)
Antibiotic susceptibility pattern of E. coli isolates

The analysis of antibiogram revealed that the most effective antibiotic was Chloramphenicol (91.30%), followed by Trimethoprim to which 86.95% of the isolates were sensitive. Also, 82.60% isolates were sensitive to Gentamicin and Ciprofloxacin, 78.26% to Ceftriaxone, 73.91% to Co-Trimoxazole and other antibiotics were still less effective. Penicillin-G showed highest resistance (100.00%) followed by Methicillin (91.30%), while 52.17% isolates were resistant to Ampicillin, 43.47% to Erythromycin and Carbenicillin, 21.73% to Tetracycline and other antibiotics were still less resistant. The antibiotic resistance profile of the E. coli isolates from milk and milk products is shown in Table 6.

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How to cite this article:

Monika Soni, Abhishek Gaurav, Bincy Joseph, S. S. Shekhawat and Subhash Chand Meena. 2019. Prevalence and Drug Resistance Pattern of *Escherichia coli* Strains Isolated from Milk and Milk Products. *Int.J.Curr.Microbiol.App.Sci.* 8(10): 1338-1346.

doi: [https://doi.org/10.20546/ijcmas.2019.810.156](https://doi.org/10.20546/ijcmas.2019.810.156)