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Occurrence of Shiga Toxin Producing *E. coli* in Milk and Milk Products collected in and around Jabalpur

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ABSTRACT

The aim of the present study was to observe the occurrence and to characterize *E. coli* isolates from various sources of milk and milk products in Jabalpur city. In total 210 milk and milk products samples were collected from different sources in Jabalpur city. For isolation of *E.coli*, the milk samples were enriched in macconkey lactose broth followed by selective plating on eosin methylene blue agar. The isolates were characterized on the basis of molecular typing and their antimicrobial profile. Out of 210 samples examined, 49 *E. coli* isolates were obtained showing an overall occurrence of 23.33% with the highest presence in raw milk (40.07%) followed by ice cream (28.57%), khoa (21.42%), *dahi* (16.66%) and flavored milk (9.52%). The molecular study with PCR revealed that a total of 55.1% (27/49) *E. coli* isolates were positive for *uspA* gene. Out of 27 *uspA* positives, 3(11.1%) and 8(29.62%) were possessing *stx1* and *stx2* respectively and 1(3.7%) had both *stx1* and *stx2* gene. Among the antibiotics tested, *E. coli* showed higher sensitivity to netillin (95.9%), gentamicin (87.7%), amikacin and chloramphenicol (77.5%), ofloxacin (71.4%), kanamycin (65.3%), norfloxacin (63.2%) and ciprofloxacin (59.1%) and resistant to nitrofurantoin (81.6%), ampicillin (75.5%), cefotaxime (71.4%), tetracycline (61.2%) and ceftazidime (55.1%). The results indicates the presence of pathogenic strains of *E. coli* in milk and milk products, which reflects poor hygienic condition and thus, the consumption of products may cause public health hazards.

Keywords: Occurrence, *E. coli*, milk, milk products

Milk is a nature's ideal and perfect food both for new born and mature human beings. Milk and milk products have high nutritive value and moisture content; therefore, these products serve as an excellent culture medium for the growth and multiplication of many kinds of microorganisms such as bacteria, rickettsia etc. *E. coli, Salmonella spp., L. monocytogenes, S. aureus, M. bovis, B. abortus* etc. are common pathogens detected in milk (Jafari *et al*., 2009; Zeinhom *et al*., 2014).

The pathogens can contaminate the milk directly from dairy animals due to udder infection or through milkers, farm environment particularly water, utensils, dust etc. Milk products during preparation or processing are contaminated with (i) equipments, which in turn are contaminated with raw milk in dairy processing plants, resulting in accumulation of pathogens and biofilms formation and subsequently contamination of processed milk products; (ii) unpasteurized milk from which the product is derived or through cross- contamination; (iii) inadequate or faulty methods of pasteurization / sterilization, packaging or storage; (iv) contaminated hands of occupational worker during handling or processing etc. These pathogens may lead to food borne diseases which are even fatal in nature (Mishra *et al*., 2017). Presence of coliform bacteria such as *E. coli* in food act as an indicator of insanitary

quality (Benkerroum *et al*., 2004) and many of the diarrhoegenic strains are known to be pathogenic in nature associated with severe diarrhea and vomition in infants and young children. Shiga toxin producing *E. coli* leads to hemolytic uremic syndrome and hemorrhagic colitis and its even 10 cells are able to precipitate diseases (Greig *et al*., 2010). As per an estimate shigatoxigenic *E. coli* causes 265,000 illness, 3,600 hospitalizations and 30 deaths in the USA every year with severe complication in children below 5 years of age (CDC, 2017). Several outbreaks have been reported by WHO (2017) in past few years in various European countries. The condition is further worsened due to development of antibiotic resistance in these pathogens. In the past few years, study on *E. coli* has also reported development of antibiotic resistance in many strains. Therefore, the present survey was conducted to study the occurrence of *E.coli* in milk and milk products and to characterize pathogenic strains with antimicrobial profiling.

MATERIALS AND METHODS

Isolation of *E. coli*

In total 210 samples of milk and milk products comprising of 42 raw milk and 168 milk products (42 each of khoa, ice-cream, flavored milk and dahi /curd) were collected from different sources like vendors, homes, dairy and retail shops in Jabalpur city in sterile bottles and poly bags. Five ml or gm of diluted sample was taken into McConkey lactose broth (MLB, Hi-Media) and incubated at 37°C for 12-24 h. The enriched inoculum (0.1 ml) was streaked on McConkey lactose agar (MLA, Hi-Media) plates and incubated at 37°C for 24 h. Lactose fermenting pink, smooth, round colonies were then streaked onto eosin methylene blue agar (EMB, Hi-Media) plates and incubated at 37°C for 24 h. Colonies showing metallic sheen were picked up and subjected to biochemical characterization by Gram's staining, motility, oxidase test, IMViC pattern according to method described by Agarwal *et al*. (2003).

Molecular characterization

The genes (*uspA*, stx1 *a*nd *stx2)* of isolated organisms were amplified using thermal cycler (Bio-Rad) as mentioned by Paton and Paton, 1998 and Osek, 2001. Template DNA for PCR reactions were prepared by boiling and snap chilling method. In brief, 1.5 ml of overnight grown brain heart infusion (BHI) broth culture was centrifuged at 10,000 g for 5 min twice, followed by washing with triple distilled water. The washed pellets were finally resuspended in 1.0 ml sterile distilled water. Then tubes were incubated in boiling water bath for 20 min followed by immediate chilling on crushed ice. The supernatant was used as template DNA after final centrifugation. PCR mixture was standardized in 25µl volume containing 5µl of purified DNA template, 2.5µl of 10 x Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8.0, 1mM DTT, 0.1 Mm EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol and 20 mM $MgCl_2$), 0.2 mM dNTP mix, 10 pmol of each forward and reverse primer, 1 unit Taq DNA polymerase and rest of fresh milli Q water followed by mixing of reaction mixture. The PCR amplification profile consisted of initial denaturation at 94°C for 5 min, followed by 35

Table 1: Details of primers used in PCR reaction

Pathogen	Gene	Primer	Product Size (bp)	
E. coli	*uspA*(F)	CCGATACGCTGCCAATCAGT	884	Osek (2001)
	uspA(R)	ACGCAGACCGTAAGGGCCAGAT		
	stx1(F)	ATAAATCGCCATTCGTTGACTAC	180	Paton and
	stx1(R)	AGAACGCCCACTGAGATCATC		
	stx2(F)	GGCACTGTCTGAAACTGCTCC	255	Paton (1998)
	stx2(R)	TCGCCAGTTATCTGACATTCTG		

cycles of denaturation at 94°C for 1 min, annealing at 50°C for *uspA*, 57°C for *stx1* and 55°C for *stx2* gene for 1 min and extension at 72°C for 1 min, with final extension for 10 min at 72°C. The amplified products were resolved on 1.5% agarose gel and visualized for documentation in gel-doc transilluminator system (Bio-Rad).

Antimicrobial assay

Antimicrobial study was performed by inoculating isolates of *E.coli* in brain heart infusion broth (BHI) at 37°C for 3 to 5 h. A sterile cotton swab was dipped into broth culture and spread across the entire agar surface of Muller-Hinton agar (MH agar, Hi Media). The petriplate was left undisturbed for 30 min and antibiotic octadisc (Hi Media) was placed with the help of sterile forceps (Bauer *et al*., 1966). The petriplates were incubated overnight at 37°C. The interpretation of results was made according to the instructions of the manufacturer.

RESULTS AND DISCUSSION

Out of 210 samples examined, 49 *E. coli* isolates were obtained showing an overall occurrence of 23.33% with the highest presence in raw milk (40.07%) followed by ice cream (28.57%), khoa (21.42%), dahi (16.66%) and flavored milk (9.52%) (Table 2 and 3).

As per FSSAI standard (2015), *E. coli* should be absent in 0.1gm samples of food. This organism is a commensal enteric bacterium and thus contaminates the environment. Their presence in food may precipitate enteric and systemic diseases. The

Table 2: Occurrence of *E. coli* in milk and milk products

Samples	Sample type	Number of samples	Number of samples positive f*or E. coli*	*Occurrence of E. coli* (%)
Milk	Raw milk	42	17	40.47
Milk products	Khoa	42	9	21.42
	Ice-cream	42	12	28.57
	Flavored milk	42	4	9.52
	Dahi	42	7	16.66
Overall occurrence		**210**	**49**	**23.33**

Table 3: Source wise occurrence of *E. coli* in milk and milk products

Sample tested	Sample source	Number of samples	Number of samples positive for *E. coli*	Occurrence of *E. coli* (%)
Raw milk	Home	14	4	28.5
	Dairies	14	5	35.7
	Vendors	14	8	57.1
Khoa	Sweet shop	14	2	14.2
	Khoa market	14	7	50.0
Ice-cream	Home	14	0	0.0
	Brand –I	14	3	21.4
	Brand –II	14	4	28.5
	Local	14	5	35.7
Flavored milk	Brand- I	14	0	0.0
	Brand –II	14	0	0.0
	Local	14	4	28.5
Dahi	Brand –I	14	1	7.1
	Brand –II	14	0	0.0
	Local	14	6	42.8

presence of *E.coli* in milk and their products is an indication of insanitary practices in the milk parlor and other places of Jabalpur city.

The findings of the present study were in agreement with Singh *et al*., 2011; Thaker *et al*., 2012; Nema *et al*., 2013; Virpari *et al*., 2013; Lubote *et al*., 2014 and Munsi *et al*., 2015. In the above studies, different author reported the presence of *E. coli* in raw milk to be around 50.0% except Singh *et al*. (2011) and Rehman *et al*. (2013) wherein they reported the presence of *E. coli* to be between 32-35%. In dairy products like ice-creams, Nema *et al.* (2013) reported the presence of 20.75% *E. coli.* They also reported 7.6% in Shrikhand, a product similar to *dahi*. Lingathurai and Vellathurai (2010) observed the presence of *E. coli* in dairy farms milk to be as high as 70%. Another similar study in dairy farms reported the presence of *E. coli* in 15.93% milk samples (Al-Zogibi *et al*., 2015).

The presence of *E. coli* in milk and products may be due to poor hygienic conditions in the farm, food processing units and retail outlets, which lead to occurrence of *E. coli*. Molecular characterization of 49 *E. coli* isolates revealed that 55.1% were positive for *uspA* (Table 4). Several bacteria had shown the presence of uspA gene under stress condition but these genes are also observed in microbes during steady-state growth in the absence of external stress. These are functional proteins and are used to differentiate *E. coli* from other gram – negative bacteria (Siegele, 2005). The occurrence of *stx1* and *stx2* had been reported time and again in food since 1982. The cattle were found to be main reservoir of shigatoxigenic *E. coli* (Rehman *et al*., 2013). *stx1* (10.5%), *stx2* (7.0%) and both *stx1 and stx2* (1.5%) were reported from different foods of animal's origin (Rehman *et al*. 2013). Altalhi and Hassan (2009) observed the presence of 3.0% *stx1* and 6.1% *stx2* in raw milk. Nema *et al*. (2013) found the presence of shiga toxin in raw milk, ice-cream, shrikhand and pasteurized milk to be 13.6%, 9.0%, 50.0% and 0.0% respectively; while Virpari *et al*. (2013) reported shiga toxin from milk and milk products to be 31.25%, 8.5% and 15.0% as *stx1*, *stx2* and both *stx1* and *stx2* respectively.

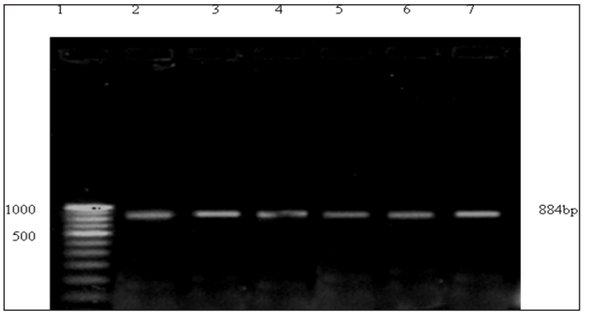

Fig. 1: Agarose gel showing amplification products of universal stress protein gene **(***uspA***)** of *E. coli* (884bp); Lane1: 100bp DNA ladder, Lane 2-7: Amplified product of *uspA* gene.

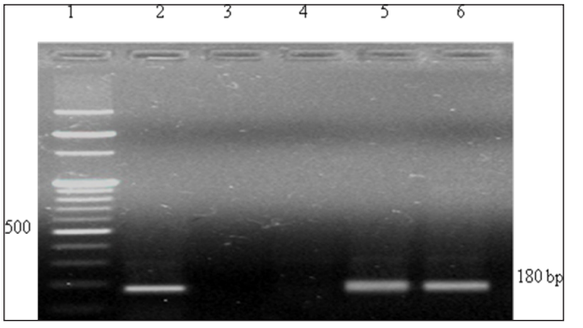

Fig. 2: Agarose gel showing amplification products of shigatoxigenic gene (stx1) of *E. coli* (180bp); Lane 1: 100bp DNA ladder, Lane 2, 5 and 6: Amplified of *stx1* gene; Lane 3 and 4: Negative control.

Table 4: Molecular characterizations of *E. coli* isolates with PCR

Isolates from various sources	*uspA*	*stx1*	*stx2*	*stx1* and *stx2*
Raw milk (n=17)	13 (76.4%)	3 (23.07%)	2 (15.3%)	1 (7.6%)
Khoa (n= 9)	6 (66.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ice-cream (n= 12)	4 (33.3%)	0 (0.0%)	1 (25.0%)	0 (0.0%)
Flavored milk (n= 4)	2 (50.0%)	0 (0.0%)	3 (75.0%)	0 (0.0%)
Dahi (n= 7)	2 (28.57%)	0 (0.0%)	2 (100%)	0 (0.0%)
Total (n=49)	**27 (55.1%)**	**3 (11.1%) (n=27)**	**8 (29.6%) (n=27)**	**1(3.7%) (n=27)**

Table 5: Antimicrobial assay of *E. coli* isolates

Name of antimicrobial agent	Pattern of antibiogram		
	Resistant	Intermediate	Sensitive
Tetracycline	30 (61.2%)	11 (22.4%)	8 (16.3%)
Ampicillin	37 (75.5%)	1 (2.0%)	11 (22.4%)
Chloramphenicol	6 (12.2%)	5 (10.2%)	38 (77.5%)
Amikacin	5 (10.2%)	6 (12.2%)	38 (77.5%)
Co-trimoxazole	28 (57.1%)	2 (4.0%)	19 (38.7%)
Kanamycin	5 (10.2%)	12 (24.4%)	32 (65.3%)
Gentamicin	3 (6.1%)	3 (6.1%)	43 (87.7%)
Streptomycin	13 (26.5%)	13 (26.5%)	23 (46.9%)
Nitrofurantoin	40 (81.6%)	6 (12.2%)	3 (6.1%)
Norfloxacin	13 (26.5%)	5 (10.2%)	31 (63.2%)
Netillin	2 (4.0%)	0 (0.0%)	47 (95.9%)
Ofloxacin	8 (16.3%)	6 (12.2%)	35 (71.4%)
Ceftaazidime	27 (55.1%)	3 (6.1%)	19 (38.7%)
Ciprofloxacin	14 (28.5%)	6 (12.2%)	35 (71.4%)
Cefotaxime	35 (71.4%)	4 (8.1)	10 (20.4)
Nalidixic acid	27 (55.1%)	12 (24.4%)	10 (20.4)

In bovine raw milk, Vendramin *et al*. (2014) observed the presence of *stx1* and *stx2* to be 28.3% and 1.9% respectively. *stx2* was found to be 67.44% among *E. coli* isolates in a dairy farm study (Al-Zogibi *et al*., 2015).

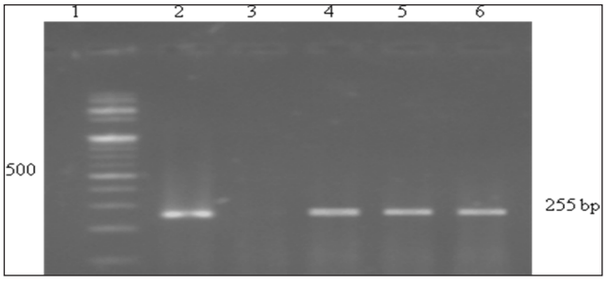

Fig. 3: Agarose gel showing amplification products of shigatoxigenic gene (*stx2*) of *E. coli* (255bp); Lane 1:100bp DNA ladder; Lane 1, 3, 4 and 5: Amplified product of *stx2* gene; Lane 2: Negative control.

The occurrence of shigatoxigenic *E. coli* in milk and milk products may be attributed to insanitary condition in environment and also cattle act as reservoir of the pathogen (CDC, 2017). The occurrence of shigatoxin may lead to serious public health hazard because of its association with hemorrhagic colitis and hemolytic uremic syndrome in humans. It is a potential food borne pathogen and can precipitate disease even with low counts viz 10-100 cells (Bandyopadhyay *et al*., 2012).

The results of the present study on antimicrobial susceptibility have been shown in Table 5. The observation revealed multidrug resistance in many isolates viz. resistance more than two antimicrobials. In the present study, 89.7% isolates were found resistant to multidrug resistant. One of the isolates revealed resistance to 15 antimicrobials. Our observations were in concurrence with many workers (Munsi *et al*., 2015; Nema *et al*., 2013). The isolates in the present study were resistant to nitrofurantoin (81.6%), ampicillin (75.5%), cefotaxime (71.4%), tetracycline (61.2%) and ceftazidime (55.1%). Resistance of *E. coli* against the ampicillin had been shown by the previous workers (Singh *et al*., 2011; Thaker *et al*., 2012; Virpari *et al*., 2013; Nema *et al*., 2013) wherein they observed the resistance to be 32.14%, 100.0% and 67.6%, respectively. Similarly, many researchers

also reported resistance against different antibiotics viz. Thaker *et al*. (2012) for oxytetracycline (43.37%), Nema *et al*. (2013) for norfloxacin (84.5%), Pant *et al*. (2013) and Reuben *et al*. (2013) for tetracycline (100%). Some workers observed higher sensitivity of *E. coli* against chloramphenicol, ofloxacin, ciprofloxacin, gentamicin and amikacin (Nema *et al*. 2013; Reuben *et al*. 2013; Virpari *et al*. 2013). Few researchers have also reported resistance problem of *E. coli* against chloramphenicol (Reuben *et al*., 2013; Pant *et al*., 2013). In our study we observed 12.2% isolates resistant against chloramphenicol which further corroborates the previous reports. The multidrug resistance problem found in our study against *E. coli* further validates the findings of Thaker *et al*. (2012), Nema *et al*. (2013) and Rehman *et al*. (2013). The antimicrobial profile of *E. coli* revealed multidrug resistance by different workers which is matter of urgent importance since these pathogens are known to transmit their resistance to other microorganisms through conjugation of their plasmids (Singh and Bist, 2013). The problems of haphazard and injudicious use of antibiotics have resulted in emergence of new antibacterial resistant isolates and are an emerging public health problem.

The study revealed insanitary quality of milk and milk products with the presence of pathogenic strains of *E. coli*, which causes fatal disease and thus, poses public health hazards. The widespread and multidrug resistance in different products is also a matter of concern because of increase virulence of such pathogen (Beceiro *et al*., 2013). Therefore, during collection, distribution and processing of milk and their products, the hygienic measures and HACCP principles should be adhered to reduce disease incidence.

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