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Molecular Characterization of *Escherichia coli* Isolated from Raw and Pasteurized Milk

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ABSTRACT

In the present study, an attempt to isolate and identify *E. coli* from raw and pasteurized milk was made. A total of 120 samples comprising of raw milk (80) and pasteurized milk (40) were processed for the isolation of *E. coli.* These 120 samples of raw and pasteurized milk samples were collected randomly from local milk vendors, milk store , milk parlour, dairy booth and retail dairies located in different parts of Jaipur city, Rajasthan. Out of 120 samples, the prevalence of *E. coli* was recorded in raw milk and pasteurised milk as samples as 31.25% (25) and 27.5% (11) respectively. In the present investigation, 12 different antibiotics were used in to obtain antibiogram for 36 isolates of *E. coli* recovered from raw and pasteurized milk samples. The analysis of antibiogram revealed that the most effective antibiotic was Enrofloxacin (83.33%), followed by Nalidixic acid (75%) and Oxytetracycline (75%) of the isolates were sensitive. Also, 72.22% isolates were sensitive to Co-trimoxazole, 63.88% to Ceftriaxone and Trimethoprim respectively and other antibiotics were less effective. Erythromycin showed highest resistance (47.22%) followed by Trimethoprim (36.11%) and cefotaxime (30.55%). Out of 36 *E. coli* isolates, 17 (47.22%) were found to have MAR index more than 0.2, thus indicating injudicious use of antibiotics. On molecular profiling, all the 36 isolates were found to be positive for *ITS* gene, *uidA*gene and *uspA* gene. Out of the 36 isolates, one (2.77%) was positive for virulence gene *(stx1).*

HIGHLIGHTS

- The isolates were highly resistant to Erythromycin, Trimethoprim and cefotaxime.
- Seventeen isolates have a MAR index more than 0.2 indicating their ability to emerge as MAR pathogen.

Keywords: Molecular Characterization *E. coli,* prevalence, raw and pasteurized milk

Milk industry is an important sector of the Indian economy particularly in poverty alleviation and employment generation. Milk production has increased at a steady rate with an average annual growth rate of 6.3% per year during last 6 years whereas world milk production is growing at the rate of 1.5% per year. Percapita availability of milk has increased from 307 grams per person per day in 2013-14 to 406 grams per person per day in 2019-2020 i.e. 32.24% (PIB2021). Fresh, non-pasteurized milk generally contains varying numbers of microorganisms, depending on the care employed in milking, cleaning and handling of milk utensils. Zoonotic pathogens present in raw milk are of great Public Health and economic significance. They also constitute a major impediment to the trade of animals

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and animal products, and thus can lead to obstruction in social and economic progress, especially in developing countries.

The habitual consumption of unpasteurized milk and its products presents considerable risk of milk-borne infections to consumers (Yakubu *et al.,* 2018) Raw milk, pasteurized milk, yoghurt and other dairy products contaminated with *E. coli,* have been the main cause of several outbreaks of milk borne disease since the 1980s and thus remain a serious health risk (Seo *et al.,* 1998). *E. coli* needs special attention, particularly in the developing countries due to poor hygienic conditions. Different studies showed that 1-5 per cent of food borne infections were related to consumption of milk and dairy products and 53 per cent of cases of food borne infections caused by contaminated cheese and entero-pathogenic *Escherichia coli* as the causative agent of 18.33 per cent of these cases (Oliver *et al.,* 2009 and Patil *et al.,* 2014).

The emergence of multiple antibiotic resistance (MDR) is the most serious threat faced by world health system. The presence of MDR pathogens in the food especially in the milk which is consumed as raw or pasteurised in many parts of the country including Rajasthan is a convenient way for these pathogens to enroute into human beings. The *E. coli* in milk might have reached the milk from animals, milkers, environment, or through post pasteurization contamination. So milk is a good medium for the carriage of various viral typesas well as multiple drug resistant *E. coli* to a large population from infants to adults.

So keeping in view of above facts and importance of microbial quality of raw milk and pasteurized milk, with emphasis on the pathogenic bacteria of Public Health importance, this study was designed to assess the prevalence of *E. coli* in raw milk and pasteurized milk. as well as study the antimicrobial resistance pattern of the pathogens and detect their ability to emerge as an MDR pathogen.

MATERIALS AND METHODS

In the present study, attempts were made to isolate and identify *Escherichia coli* from raw and pasteurized milk sold in Jaipur city of Rajasthan. The study was conducted at Department of Veterinary Public Health and Epidemiology and CDSRZ Laboratory, PGIVER Jaipur. A total of 120 samples of raw and pasteurized milk from Jaipur city. The samples were processed as per the standard microbiological techniques isolation was done by selective enrichment in broth and plating on MacConkey agar (HiMedia) Edward and Ewing (1972) and Singh *et al.* (2018) with certain modifications. 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 (Hitchins *et al.,* 2001; Rai *et al.,* 2020 and Sultana *et al.,* 2021).

Polymerase chain reaction for the detection of *ITS, uidA*, *uspA* gene and *stx1* gene

The biochemically confirmed isolates were further confirmed by PCR which was standardized by targeting the virulence gene of *E. coli* isolates recovered from raw and pasteurized milk. The primers used in the study are listed in Table 1. The template DNA was prepared as per the method of HiMedia TM Bacterial Genomic DNA Purification Kit. Molecular detection of *E. coli* all the isolates used for performing DNA isolation as described by using Nucleopore genomic DNA fungal/bacterial mini kit procedure. The molecular characterization was done by using PCR. 36 isolates of *E. coli* revealed the presence of *ITS, uidA, uspA* and *stx1* gene.

Antibiotic susceptibility tests

The antibiotic susceptibility tests were performed as per method described by Bauer *et al.* (1966) with certain modification to find out the antibiotic resistance pattern of all *E. coli* isolates. *In vitro* antibiotic sensitivity test of the isolates was conducted by paper disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India). Isolates were subjected to antimicrobial sensitivity tests against 12 antibiotics Antimicrobial discs used were Enrofloxacin (10 mcg), Nalidixic acid (30mcg), CoTrimoxazole (25 mcg), Trimethoprim (10mcg), Erythromycin (10 mcg), Azithromycin (15 mcg), Ceftriaxone (30 mcg), Cefotaxime (30 mcg), Ampicillin (25 mcg), Amoxyclav (30 mcg), Oxytetracycline (30 mcg) and Streptomycin (10 mcg) was used for resistance confirmation.

Table 1: The oligonucleotide primers used for detection of *ITS, uidA, uspA* and *stx1*, gene

Sl. No.	Oligo name	Sequence (5 -3)	Size of amplified product (bp)	Reference
1	*ITS*	**F**- GCT TGA CAC TGA ACA TTG AG	662	Khaled *et al.* (2010)
		R- GCA CTT ATC TCT TCC GCA TT		
2	uidA	**F**-GCG TCT GTTGACTGGCAGGTGGTGG	510	Johnson *et al.* (2017)
		R-GTTGCCCGCTTCGAAACCAATGCCT		
3	*uspA*	**F**-CCGATACGCTGCCAATCAGT	884	Osek, (2001)
		RACGCAGACCGTAAGGGCCAGAT		
4	*Stx1*	**F**-CAGTTAATGTGGTGGCGAAG	894	Hazarika *et al*. (2007)
		R-CTGCTAATAGTTCTGCGCATC		

F = Forward, R = Reverse.

Table 2: Quantity and concentrations of various components used in PCR Master Mix for detection of *ITS, uidA, uspA* and *stx1*, gene

Sl. No.	Components	Quantity	Concentration
1	DNase-RNase free water	7.50 µl	—
2	2X PCR master mix	12.50 µl	2X
3	Forward Primer (10 pmol/µl)	1.00 µl	10 pmol
4	Reverse Primer (10 pmol/µl)	1.00 µl	10 pmol
5	DNA Template	3.00 µl	—
	Total	**25.00 µl**	**—**

Table 3: Steps and conditions of thermal cycling for different primer pairs in PCR

Primers	Cycling conditions				
(Forward and Reverse)	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
ITS(F)	96 ºC	96 ºC	57 ºC	72 ºC	72 ºC
ITS(R)	4 minutes	30 sec	30 sec	30 sec	7 minutes
Repeated for 40 cycles					
uidA(F)	94 ºC	94 ºC	67 ºC	72 ºC	72 ºC
uidA(R)	5 minutes	1 minute	1 minute	1.5 minutes	5 minutes
Repeated for 30 cycles					
uspA (F)	94ºC	94 ºC	55 ºC	72 ºC	72 ºC
uspA(R)	5 minutes	1 minute	1 minute	2 minutes	5 minutes
Repeated for 30 cycles					
stx1 (F)	94ºC	94 ºC	55 ºC	72ºC	72 ºC
stx1(R)	5 minutes	1 minute	1 minute	1.5minutes	5 minutes
Repeated for 30 cycles					

RESULTS AND DISCUSSION

The *E. coli* isolates were stained with Gram's staining method, and then characterised using biochemical tests such as oxidase, catalase, IMViC pattern (indole production, Methyl Red (MR) test, Voges-Proskauer (V.P) test, citrate utilisation (on Simmon's citrate medium), urea hydrolysis, production of H_2S on TSI agar. The isolates were identified as Gram negative bacilli by Gram's staining. The isolates were catalase positive, oxidase negative, + + - - IMViC patterns, and H_2S production negative on TSI agar. All the isolates which produced bright pink colonies on MacConkey agar Fig. 1 and colonies with a characteristic metallic sheen on EMB agar, mentioned in Fig. 2. Out of 120 samples examined, 36 isolates were obtained showing an overall prevalence of 30.00 per cent, as depicted in table 4. The highest prevalence was observed in raw milk (31.25%), mentioned in table 4. Similar prevalence were obsereved by such as 31.3 per cent by Tangri and Chatli (2014) and 32.14 per cent by Singh *et al.* (2011). Higher prevalence of *E. coli* was reported 52 per cent by Virpari *et al.* (2013).

Table 4: Prevalence of *E. coli* in raw and pasteurized milk

Sl. No.	Sample	Number of sample	Number of positive sample	Prevalence of *E. coli* (%)	Over all prevalence
1	Raw milk	80	25	31.25%	
2	Pasteurized milk	40	11	27.5%	30

Fig. 1: Growth on MacConkey agar plate

Pink lactose fermenting *E. coli* on Macconkey agar

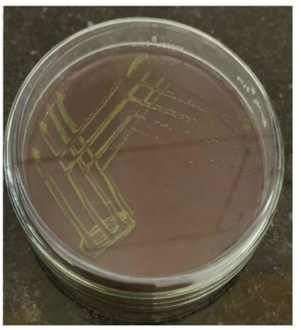

Fig. 2: Growth on EMB agar plate

E. coli produces greenish metallic sheen on EMB agar

Molecular confirmation of test isolates

The biochemically confirmed isolates were further confirmed by PCR which was standardized by targeting the virulence gene of *E. coli* isolates recovered from raw and pasteurized milk.

Detection of *ITS, uidA, uspA* and *stx1*, gene

Screening of samples for the presence of *ITS, uidA, uspA* and *stx1*, gene was done by PCR (Fig. 3,4,5 and 6).

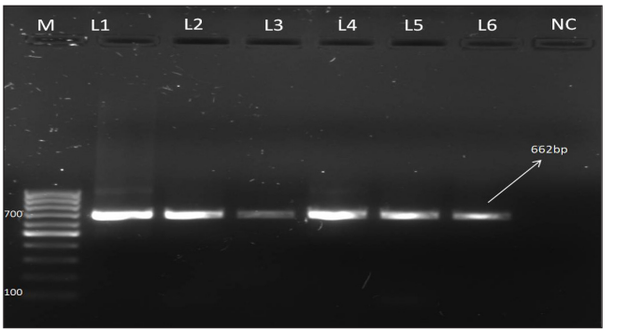

Fig. 3: Agarose gel showing PCR amplified product (662 bp) for *ITS* gene in the test isolates. M = 100bp DNA ladder, positive samples (L1 = 10, L2 = 12, L3 = 74, L4 = 78, L5 = 111, L6= 115, NC = negative control)

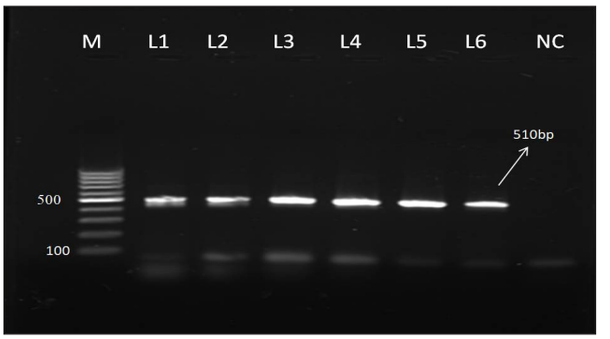

Fig. 4: Agarose gel showing PCR amplified product (510 bp) for *uidA* gene in the test isolates. M =100bp DNA ladder, positive samples (L1 = 10, L2 = 12, L3= 74, L4 = 78, L5 = 111, L6 = 115, NC = negative control)

Out of 120 *E. coli* isolates recovered from raw and pasteurized milk. Thirty six isolates (30%) were found to be positive for *ITS, uidA* and *uspA* gene. While, out of 120 *E. coli* isolates from raw and pasteurized milk only 1

isolates (2.77%) were found to be positive for *stx1* gene. Our finding is somewhat similar to Bradley (2002) for *ITS* gene who reported 34.7% for *E. coli* infection, Balaji *et al*. (2018) and Mussa and Al-Mathkhury (2018) reported *uspA* gene as 100% and 96%, respectively. Mohammadzadeh *et al.* (2017) founded *uidA* gene 100% in all biochemically confirmed *E. coli* isolates.

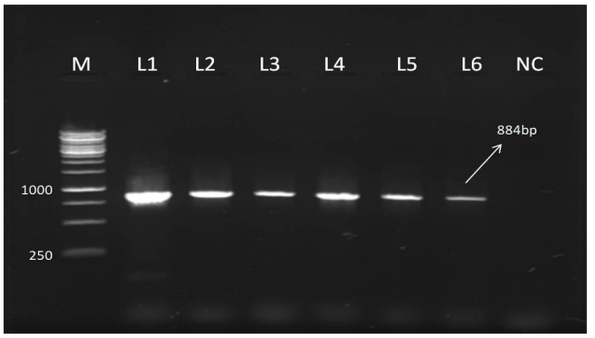

Fig. 5: Agarose gel showing PCR amplified product (884 bp) for *uspA* gene in the test isolates. M = 100bp DNA ladder, positive samples (L1 = 10, L2 = 12, L3 = 74, L4 = 78, L5 = 111, L6 = 115), NC = negative control

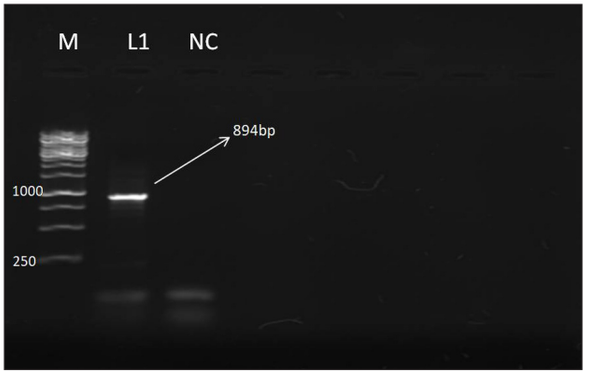

Fig. 6: Agarose gel showing PCR amplified product (894 bp) for *stx1* gene in the isolates. M = 100bp DNA ladder, positive samples (raw milk = 18) NC = negative control

Table 5: Prevalence of *E. coli* of ITS, *uidA, uspA* gene and *stx1* gene from raw and pasteurized milk

Name of the gene	Positive isolates
ITS	36 isolates (100%)
UidA	36 isolates (100%)
UspA	36 isolates (100%)
stx1	1 isolates (2.77%)

Antibiotic susceptibilty test of *E. coli* isolates

In present study among 36 *E. coli* isolates from raw and pasteurized milk the highest resistance was observed against isolates were resistant to Erythromycin 17 (47.22%) followed by Trimethoprim 11 (30.55%), Cefotaxime 11(30.55%), Ampicillin 10 (27.77%), Ceftriaxone 9 (25%) and Nalidixic acid 8 (22.22%) respectively. The least resistance was observed against 6(16.66%) for Azithromycin, Ampicillin and Amoxyclav respectively. Co-Trimoxazole and Streptomycin showing against 4 (11.11%) respectively. No resistance was observed against Enrofloxacin while the highest sensitivity was recorded for Enrofloxacin 30 (83.33%), mentioned in table 6, 7 and 8. Similarly, 40.63 per cent resistance were observed by Rahman *et al.* (2017), Trimethoprim 36.36 per cent by Ababu *et al.* (2020) from raw milk of dairy cattle in Holeta district, central Ethiopia. Highest resistance against Cefotaxime was recorded 100 per cent by Mohammed *et al.* (2021). *E. coli* isolates were resistant to several antibiotics like Erythromycin, Amoxycillin and Oxytetracycline. These findings are in agreement with Munsi *et al.* (2015) from milk samples of different locations in Bangladesh.

Table 6: Antibiogram assay of *E. coli* isolates recovered from raw milk samples

Name of antibiotics	Antibiotic Sensitivity Pattern		
	Sensitive	Intermediate	Resistant
Enrofloxacin	21 (84%)	4 (16%)	0 (0.00%)
Nalidixic acid	16 (64%)	0 (0.00%)	9 (36%)
Co-Trimoxazole	17 (68%)	4 (16%)	4 (16%)
Trimethoprim	17 (68%)	0 (0.00%)	8 (32%)
Erythromycin	6 (24%)	7 (28%)	12 (48%)
Azithromycin	10 (40%)	10 (40%)	5 (20%)
Ceftriaxone	17 (68%)	2 (8%)	6 (24%)
Cefotaxime	7 (28%)	11 (44%)	7 (28%)
Ampicillin	15 (60%)	4 (16%)	6 (24%)
Amoxyclav	11 (44%)	4 (16%)	5 (20%)
Oxytetracycline	21 (84%)	1 (4%)	3 (12%)
Streptomycin	15 (60%)	6 (24%)	4 (16%)

Table 7: Antibiogram assay of *E. coli* isolates recovered from pasteurized milk samples

Name of antibiotics	Antibiotic Sensitivity Pattern		
	Sensitive	Intermediate	Resistant
Enrofloxacin	9 (81.82%)	2 (18.18%)	0 (0.00%)
Nalidixic acid	10 (90.91%)	1 (9.09%)	0 (0.00%)
Co-Trimoxazole	8 (72.73%)	3 (27.27%)	0 (0.00%)
Trimethoprim	8 (72.73%)	0 (0.00%)	3 (27.27%)
Erythromycin	2 (18.18%)	4 (36.36%)	5 (45.45%)
Azithromycin	2 (18.18%)	6 (54.55%)	3 (27.27%)
Ceftriaxone	6 (54.55%)	2 (18.18%)	3 (27.27%)
Cefotaxime	1 (9.09%)	5 (45.45%)	4 (36.36%)
Ampicillin	6 (54.55%)	1 (9.09%)	4 (36.36%)
Amoxyclav	7 (63.64%)	3 (27.27%)	1 (9.09%)
Oxytetracycline	6 (54.55%)	2 (18.18%)	3 (27.27%)
Streptomycin	6 (54.55%)	5 (45.45%)	0 (0.00%)

Table 8: Antibiogram assay of *E. coli* isolates recovered from raw and pasteurized milk samples

Sl. No.	Name of antibiotics	Antibiotic Sensitivity Pattern		
		Sensitive	Intermediate	Resistant
1	Enrofloxacin	30(83.33%)	6(16.66%)	0(0.0%)
2	Nalidixic acid	27(75%)	1(2.77%)	8(22.22%)
3	Co-Trimoxazole	26(72.22%)	6(16.66%)	4(11.11%)
4	Trimethoprim	23(63.88%)	0(0.0%)	13(36.11%)
5	Erythromycin	12(33.33%)	7(19.44%)	17(47.22%)
6	Azithromycin	13(36.11%)	17(47.22%)	6(16.66%)
7	Ceftriaxone	23(63.88%)	4(11.11%)	9(25%)
8	Cefotaxime	8(22.22%)	17(47.22%)	11(30.55%)
9	Ampicillin	24(66.66%)	2(5.55%)	10(27.77%)
10	Amoxyclav	23(63.88%)	7(19.44%)	6 (16.66%)
11	Oxytetracycline	27(75%)	3(8.33%)	6 (16.66%)
12	Streptomycin	23(63.88%)	9(25%)	4(11.11%)

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