Prevalence and Risk Factors of β-Lactamase Genes of Extended-Spectrum β-Lactamases-Producing *Escherichia coli* From Dairy Farm Environments of Haryana, India

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ABSTRACT: Presence of extended-spectrum β-lactamases (ESBL)-producing *Enterobacteriaceae* in the dairy farm environment and food chain could be a possible interface for the exchange of antimicrobial resistance genes between humans and animals. A total of 600 samples comprised of raw bovine milk, faeces, feed, environmental swabs and water samples from 20 different bovine dairy farms in and around Hisar city, Haryana, India were analysed for presence of ESBL encoding genes. Out of 240 isolates of *Escherichia coli* obtained, 74 isolates were found to be ESBL producers. Maximum number of ESBL isolates were found from faeces (40.5%) followed by raw milk (37.8%) and environmental swabs (17.5%). Most of the ESBL *E. coli* isolates were sensitive to chloramphenicol (82.4%) and gentamicin (77.0%) antibiotics. The bla_{CTX-M} gene was found to be most prevalent (52.0%) followed by bla_{TEM} (9.45%) while bla_{SHV} gene alone was not detected in any sample by simplex PCR. However, the co-expression of $bla_{CTX-M} + bla_{TEM}$ (21.6%) and $bla_{CTX-M} + bla_{SHV}$ (4.05%) genes were also observed. The housing system, milking method and the hygienic mangement practices followed at farm level are found to be significant risk factors of ESBL-producing *E. coli* in dairy farms of Haryana.

KEYWORDS: Haryana, bovine dairy farms, antimicrobial resistance, ESBL ***E. coli***, CTX-M, synergy test

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Introduction

Antibiotics of the β-lactam group are the most commonly used antibiotics globally for treating bacterial infections. Extended-spectrum β-lactamases (ESBL) are enzymes that can hydrolyse the β-lactam rings of antibiotics. Antibiotic resistance (AMR) is a global issue currently and the presence of ESBL producing *Enterobacteriaceae* in the environment and food chain could be a possible interface for the exchange of resistance genes between humans and animals.[1] *Escherichia coli* can serve as a sentinel organism for checking the development of AMR because of frequent exposure to antibiotics and subsequent pressure for formation of resistant strains.[2] *Enterobacteriaceae* containing genes encoding for extended spectrum β-lactamases have emerged and spread quickly in recent years.[3] *Enterobacteriaceae* acquire ESBL by mutation or by plasmid-mediated horizontal gene transfer. While the ESBL enzymes of the bla_{CTX-M} family are originated from environmental bacteria, the bla_{TEM} and bla_{SHV} families of ESBL enzymes are mutant variants of well-known plasmid-mediated β-lactamases. The most common ESBL variations might show temporal shifts as well as geographical differences.[4] The prevalence of β-lactamases, has rapidly increased during the past few years, particularly those of the CTX-M enzymes. Prior to the year 2000, infections with ESBL-producing *Enterobacteriaceae* harbouring bla_{TEM} and bla_{SHV} genes were the most common, however, currently bla_{CTX-M} is so far the most common gene in ESBL producer strains.

Dairy farming involves a high degree of interaction between animals, humans and the environment. The milking personnel have direct interaction with the animal and the surroundings due to various husbandry activities. The bovine faeces are using as manure/soil amendment to crops and vegetables in Indian villages, which increases the possibility of transfer of antibiotic resistant genes to environment inadvertently. Due to the significant interactions at the human-dairy interface, there have been reports of evidence of AMR in food, animals, humans and the environment.[5] Therefore, the disseminaton of ESBL *E. coli* within the ecosystem which is linked to bovines are of serious concern with regard to One Health approach.[6]

AMR surveillance and monitoring analyzes and records the changes and trends in antibiotic resistant microbial populations and resistant factors such as AMR genes and resistance mechanisms. It is crucial for preventing antibiotic resistant bacteria from emerging and spreading among animals, humans and the environment. Data from surveillance systems improve our knowledge of the multifaceted AMR epidemiology. The information gathered is essential to control infections, formulating policies and measures to decrease AMR. Therefore, this

study was designed to know about the presence of ESBL producing *E. coli* in the dairy farm environment of Haryana, India. The antibiogram, ESBL encoding genes and the risk factors associated with the ESBL production were also analysed.

Materials and Methods

Collection of samples

A total of 600 samples comprised of 400 raw bovine milk, 100 faeces, 40 feed, 40 environmental swabs and 20 water samples from 20 different bovine dairy farms in and around Hisar city, Haryana, India were collected under aseptic conditions in sterile containers. The samples were collected from different villages of Hisar *viz.* Village Post Office (V.P.O.) Dabra, Mirkan, Ladwa, Satrod, Niayana, Kamiri and from Sirsa city. Five apparently healthy lactating animals from each farm were selected randomly and included in the study. From each animal, raw milk samples were collected into sterile tubes from four quarters (10 ml each) separately. However, the faecal samples were collected by per rectal method. Two samples of feed and one sample of water also collected from each farm. Sterile cotton swabs moistened with normal saline were used for sampling of environment and from each farm, 2 such samples were taken from clean floor or wall of farms. The details of farms such as managemental practice, milking method, hygienic condition, housing system, water source, use of antimicrobial medicines at farm were collected in the questionnaire designed. All the samples were labelled properly and transported in ice box to the College Central Laboaratory, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana for bacteriological analysis. The ethical approval was obtained from the competent authority (IAEC/LUVAS/28/20).

Isolation and identification of E. coli

All the samples except faeces were pre enriched in Buffered Peptone Water (BPW) (1:10 ratio) at 37°C for 18 hours, while a loopful of faecal sample was directly streaked onto MacConkey agar (MLA). From BPW, a loopful of enriched inoculum was streaked onto MLA and incubated at 37°C for 18 to 24 hours and a single colony was obtained. The lactose fermenting pink to red colonies were further streaked onto Eosin Methylene Blue agar (EMB) and incubated overnight at 37°C. The dark centred colonies with metallic sheen were considered as *E. coli* presumptively. These isolates were further characterized by Gram staining and biochemical reactions *viz.* IMViC (Indole, Methyl red, Voges Proskauer, Citrate), catalase and oxidase tests.[7] All the bacteriological media and reagents used for isolation were procured from Hi Media laboratories Pvt. Ltd, Mumbai, India.

Phenotypic detection of ESBL production

The *E. coli* isolates were checked for ESBL production on HiCrome ESBL agar (Hi Media, Mumbai, India) and incubated at 37°C for 24 hours. The colonies with purple colour were tentatively identified as ESBL producer. The ESBL producing isolates were further verified by Double-Disc Synergy Test (DDST) using ceftazidime (CAZ-30 µg) and ceftazidime with clavulanic acid (CAC-30/10 µg) as well as cefotaxime (CTX, 30 µg) and cefotaxime-clavulanate (CEC, 30/10 µg) discs. Briefly, *E. coli* isolates were inoculated into nutrient broth and incubated at 37°C. The bacterial culture with a turbidity equivalent to 0.5 Mac Farland standard unit was inoculated onto Mueller Hinton Agar (MHA) plates by spread plate method. The antibiotic discs were placed on the inoculated MHA plates at a distance of 20 mm apart and incubated overnight at 37°C. The inhibition zone diameter was measured for each antibiotic disc and its respective clavulanic acid containing discs. A difference of ⩾5 mm in the presence of clavulanic acid when compared to its absence was considered as positive for the production of ESBL.[8]

Determination of minimum inhibitory concentration

All the isolates which were positive for ESBL production on HiCrome ESBL agar were subjected to Minimum Inhibitory Concentration (MIC) test using MIC strips (EM132, Hi Media laboratories Ltd., Mumbai). The ESBL MIC (MIX+/MIX) strip was placed in the centre of MHA plates. The MIC values were noted at the point where ellipse of inhibition intersects the MIC scale on the strip. A ratio of MIX/MIX+ ⩾ 8 or ellipse of inhibition intersecting only on MIX+ side was considered as ESBL positive isolate.

Antimicrobial susceptibility testing

All the ESBL positive *E. coli* isolates obtained from various samples were subjected to antimicrobial susceptibility test according to CLSI guidelines.[8] Cultures of all ESBL *E. coli* isolates were grown in nutrient broth upto 0.5 Mac Farland standard unit and tested against 16 antibiotics from 6 different classes on MHA plates. The antibiotics included were amikacin (30 µg), ampicillin (10 µg), amoxicillin (10 µg), cefoperazone (75 µg), ceftazidim (30 µg), cefotaxime (10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), enrofloxacin (10 µg), gentamicin (10 µg), kanamycin (30 µg), levofloxacin (5 µg), moxifloxacin (5 µg), neomycin (30 µg), oxytetracycline (30 µg) and streptomycin (10 µg).

Molecular characterization of the isolates

The phenotypically confirmed *E. coli* isolates were screened for specific genetic elements by molecular method. DNA extraction of the isolates was carried out by using QIAamp DNA blood mini kit (QIAGEN, Germany). *E. coli* isolates were confirmed by PCR targeting *uidA* gene using published primers.[9] The reaction mixture was prepared using 12.5 µl of master mix (Promega, USA), 1 µl of each primer (10 µm), 2 µl DNA

Table 1. Details of primers and thermal profiling of genes.

GENE	PRIMER SEQUENCE	EXPECTED AMPLICON SIZE (BP)	DENATURATION	ANNEALING	EXTENSION	REFERENCE
*uid*A	F5′-TGGCAGGTGGTGGCAAATGGTGGTG-3′	200	94°C, 1 min	56°C, 1 min	72°C, 1 min	Deb et al[9]
	R 5′-CCGACGCGCAGCGGGTAGATAT-3′					
bla_{CTX-M}	F 5′-CGCGGTGCTGAAGAAAAGTG-3′	475	95°C, 30 s	60°C, 30 s	72°C, 30 s	
	R 5′-GCCGGTTTTATCCCCCACAA-3′					
bla_{TEM}	F 5′-ATGAGTATTCAACATTTCCG-3′	867	95°C, 1 min	55°C, 1 min	72°C, 1 min	Sivakumar et al[10]
	R 5′-CTGACAGTTACCAATGCTTA-3′					
bla_{SHV}	F 5′-TCGCCTGTGTATTATCTCCC-3′	768	94°C, 30 s	52°C, 30 s	72°C, 30 s	
	R 5′-CGCAGATAAATCACCACAATG-3′					

template and nuclease free water to make the final volume up to 25 µl. The *E. coli* isolates were also investigated for the presence of β-lactamase (*bla*) encoding genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV})[10] by simplex PCR. The ATCC strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 strains were used as negative and positive control strains, respectively. The details of primers used in this study and the thermal profiling for different genes are given in Table 1. The amplified product was resolved on electrophoresis gel (1.5%) containing ethidium bromide (Sigma, USA) at a concentration of 0.5 µg/ml and the gel was run at 75 V for 1.5 hours using submarine horizontal electrophoresis system (Genetix Biotech Asia Pvt. Ltd, New Delhi) in 1× TAE buffer. The amplified PCR product was visualized using gel documentation system (Azure Biosystems, USA) and compared with the 100 bp DNA ladder (Promega, USA) as size marker.

Statistical analysis

The farm data were analysed to find out the risk factors associated with presence of ESBL *E. coli* in dairy farms. Statistical analysis was done using chi-squared test at 95% confidence interval (IBM SPSS statistics version 21, New York). The difference was considered as statistically significant at $P \leqslant .01$ between variables.

Results and Discussion

Phenotypic characterization of ESBL E. coli

Out of 600 samples, a total 246 samples were shown pink colour colonies on MLA and greenish metallic sheen on EMB agar was observed from 240 samples. These colonies were biochemically characterized by IMViC tests and Gram's staining. Pink coloured rods were observed upon Gram's staining. All the isolates were positive for Indole and Methyl red tests. They were negative for Voges-Proskauer and Citrate test. All the phenotypically positive *E. coli* isolates were shown the presence of *uid*A gene (Figure 1).

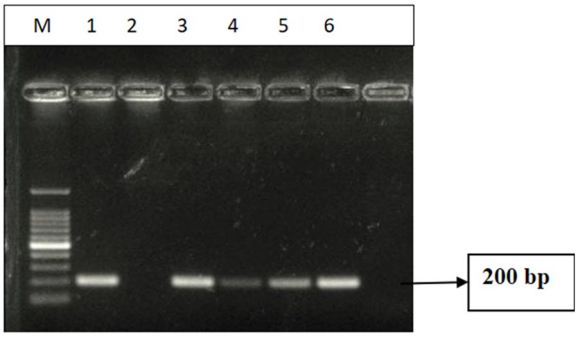

Figure 1. Agarose gel showing *uid*A gene amplified from *E. coli* isolates.
Lane M – 100 bp DNA ladder.
Lane 1 – Positive control.
Lane 2 – Negative control.
Lanes 3 to 6 – Samples.

Out of 240 isolates of *E. coli*, 74 (12.3%) isolates were found to be ESBL producers based on the growth on HiCrome ESBL agar (Figure 2). Maximum number of ESBL isolates were obtained from faeces (40.5%) followed by raw milk (37.8%), environmental swabs (17.5%), water (2.9%) and feed (1.3%) samples. All the isolates were given positive Double-Disc Synergy Test for CTX/CEC combination, but 91.89% isolates were found to be positive for CAZ/CAC combination (Figure 3), thus confirmed the production of ESBL in these isolates phenotypically. All the isolates showed zone of inhibition only on MIX+ side as it contained clavulanic acid and no zone of inhibition was found on MIX side (Figure 4). The MIC values of these isolates were noted and were ranged from 0.25 to 0.048 (Figure 5).

There is a wide variation in detection of ESBL *E. coli* in raw milk from different States of India. India is primarily divided into 6 physiographic areas, each of which has a distinct climate and culture. The husbandry methods also differ significantly from east to west and north to south. There are several factors that cause variations in the incidence of extended-spectrum beta-lactamases in different States and zones of India. The probable reason of these variations could include the sampling

Figure 2. ESBL producing *E. coli* on HiCrome ESBL agar.

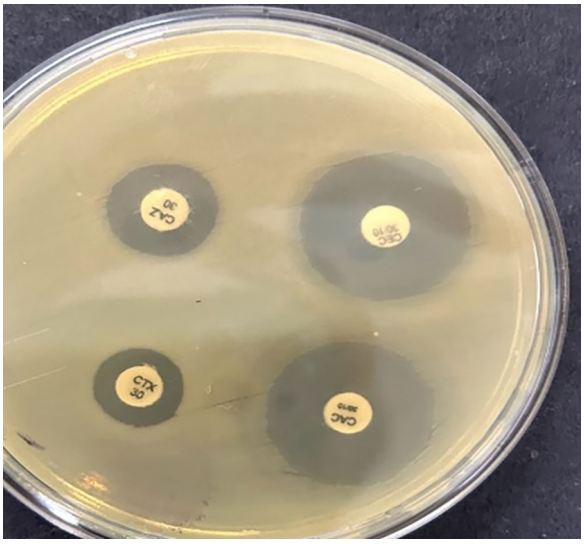

Figure 3. Double disc synergy test of ESBL *E. coli*.

period, geographic location, antibiotic class and frequency of use in various zones, virulence factors, species variances, AMR patterns of each region *etc*. The meta-analysis study from India reported difference in ESBL prevalence percentages from north, east, south and central zones.[11] Comparable to the current investigation, 37.0% and 40.0% of ESBL *E. coli* from the raw milk and faeces, respectively is reported from Chhattisgarh, India.[12] There is no report available regarding the presence of ESBL *E. coli* from Indian dairy farm environments. However, the results were in agreement with studies of foreign countries. A prevalence of 10.0% and 5.0% of ESBL-producing *E. coli* from the animal drinking water and feed, respectively is reported in Indonesia.[13] An occurrence of 28.6% ESBL *E. coli* from the environmental samples in Egypt is reported.[14] A very high prevalence of 88.7% was noticed in Chiang Mai from the waste water of dairy farms[15] and 13.0% ESBL *E. coli* occurrence from environmental samples.[16]

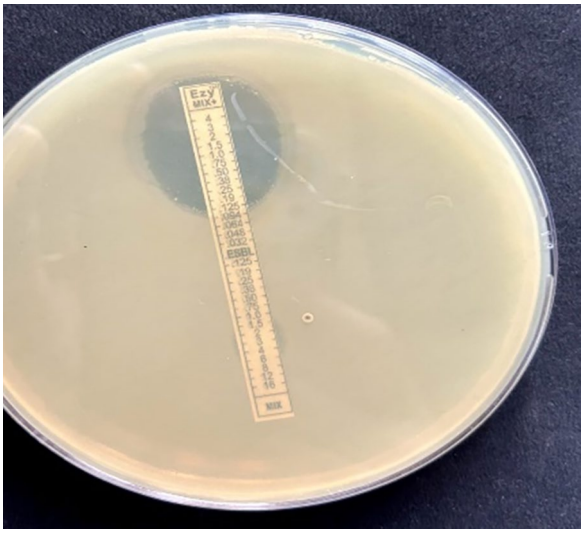

Figure 4. Minimum inhibitory concentration of ESBL *E. coli*.

Antimicrobial susceptibility pattern

Most of the ESBL *E. coli* isolates were sensitive to chloramphenicol (82.4%) followed by gentamicin (77.0%) in antibiotic susceptibility test. These antibiotics could be a alternative drug of choice for treating the infections caused by ESBL *E. coli*. However, the lowest resistance to these antibiotics seems to be associated with the restricted usage along with the reduced preference of these drugs in the treatment of infections. Complete resistance (100.0%) was observed against cephalosporins class of antibiotics. This can be due to the production of β-lactamases which destroy the β-lactam rings of these antibiotics. A very high level of resistance was observed for fluoroquinolones also. This may be due to the co-existence of ESBL with plasmid-mediated quinolone resistance genes.[17] The isolates from raw milk and faeces were 14.2% and 6.6% sensitive to enrofloxacin, respectively, while all the isolates of environmental swabs were 100% resistant. Similar findings were reported in other studies as well.[18,19] All the isolates of ESBL *E. coli* were found as multidrug resistant, with resistance to 3 or more antibiotics from 3 different antibiotic classes.[20] The antibiotic sensitivity pattern of isolates from various samples is given in Figure 6.

Molecular characterization of ESBL E. coli

Presence of any one or more β-lactamase genes *viz.* $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} were detected in 65 isolates, whereas 9 isolates were negative for all the β- lactamase genes investigated in this study. The $bla_{CTX\text{-}M}$ gene was detected in 39 isolates (52.0%) and bla_{TEM} in 7 (9.45%) isolates. However, no sample possessed bla_{SHV} gene alone by simplex PCR. The co-expression of genes $bla_{CTX\text{-}M} + bla_{TEM}$ was observed in 16 isolates (21.6%) and $bla_{CTX\text{-}M} + bla_{SHV}$ in 3 (4.05%) isolates. The gene combinations of $bla_{TEM} + bla_{SHV}$ as well as $bla_{CTX\text{-}M} + bla_{TEM} + bla_{SHV}$ were not detected in this study. The PCR

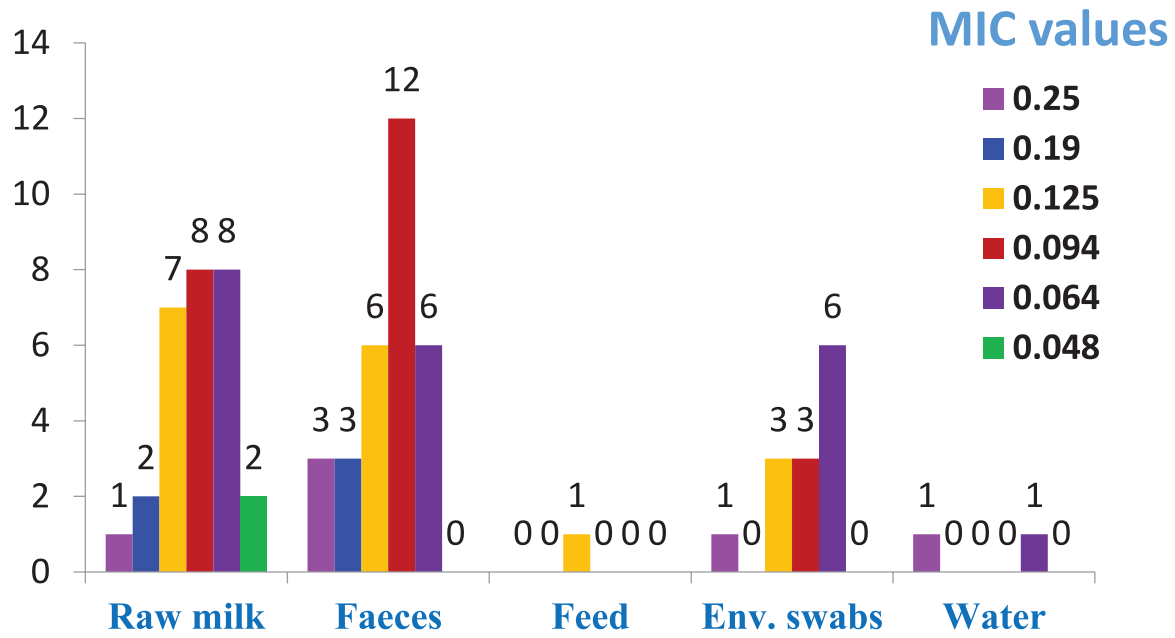

Figure 5. MIC values of ESBL *E. coli* isolates.

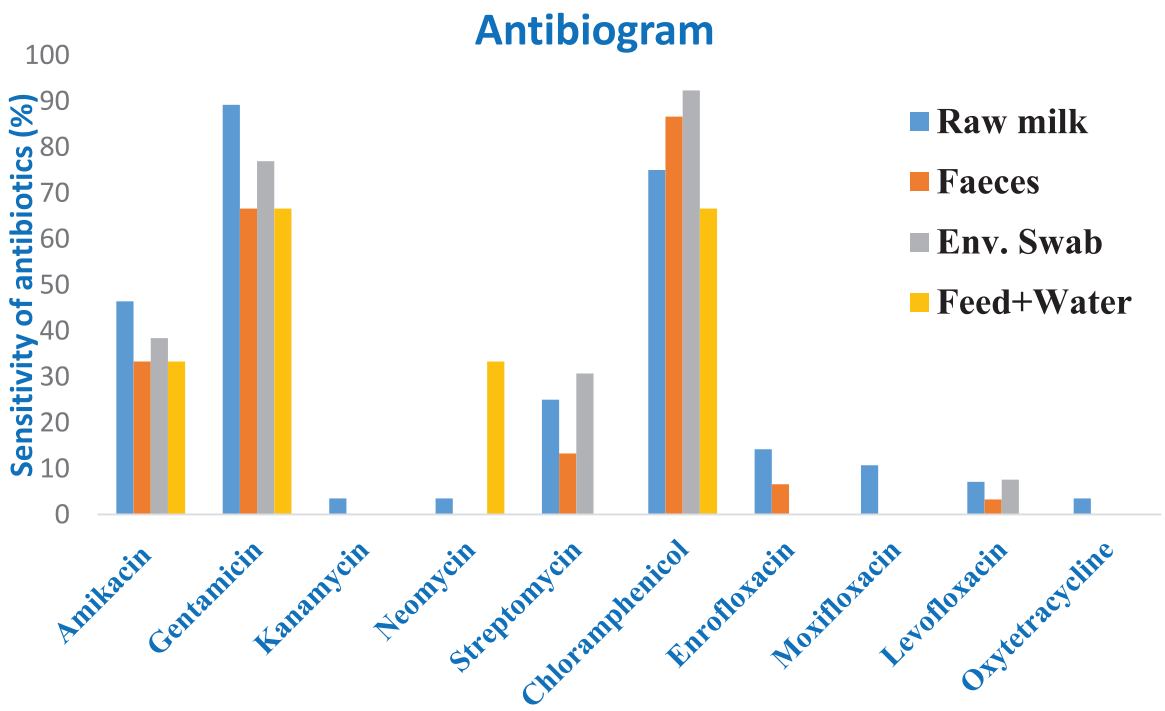

Figure 6. Antibiotic sensitivity pattern of ESBL *E. coli* isolates.

amplified products of *bla* genes and the source wise distribution of β-lactamase genes are given in Figures 7 to 9.

Concerns have been raised in both human and veterinary medicine over the swift expansion and global dissemination of *bla* genes in ESBL *E. coli*. These genes can be transmitted to humans and animals, through the bovine milk, faeces and farming environments. It has been discovered that plasmids carrying *bla* genes are capable of transferring these determinants to other commensal bacteria or pathogens.[21] There have been varying reports nationwide on the presence of bla_{CTX-M} in isolates of *E. coli* derived from milk. A higher carriage (54.54%) of bla_{CTX-M} gene from West Bengal[22] is found. However, a low prevalence (2.09% and 3.12%) of bla_{CTX} in milk is also reported from Chhattisgarh and Orissa, respectively.[12,23]

The findings of the current study regarding the detection of bla_{CTX-M} in faeces were comparable to that from Assam, who reported 28.57% bla_{CTX-M} in *E. coli* from cattle faeces.[24] Comparable to the current investigation, 19.0% prevalence of bla_{CTX-M} gene from feed of cattle in Bhopal is reported.[25] A prevalence rate of 5.4% and 93.4% is found for bla_{CTX-M} gene

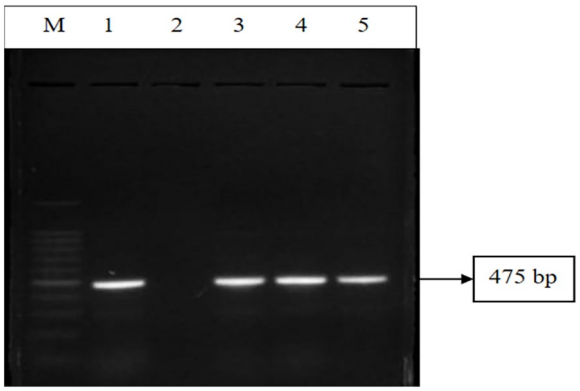

Figure 7. Agarose gel showing *bla*$_{CTX-M}$ gene amplified from *E. coli* isolates.

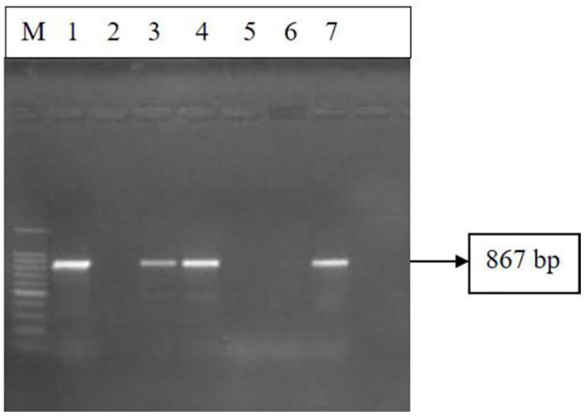

Figure 8. Agarose gel showing *bla*$_{TEM}$ gene amplified from *E. coli* isolates.

from the dairy farm environment, respectively.[26,27] A 95.9% prevalence of *bla*$_{CTX-M}$ from raw milk and environmental samples is recently reported.[28]

The current study's percent prevalence for *bla*$_{TEM}$ was higher than that of previous reports. A low prevalence of 3.66% was reported for *bla*$_{TEM}$ in raw milk.[29] A comparable prevalence of 7.2% of *bla*$_{TEM}$ in raw milk isolates as of our study is also available.[30] In another study, 28.57% isolates were harbouring *bla*$_{TEM}$ in raw milk samples.[31] The *bla*$_{SHV}$ gene was found to be present in only 3 isolates obtained from faecal samples. Analogous reports can be found from North Macedonia[32] with 2.56% occurrence of *bla*$_{SHV}$ gene in faeces. There are reports of absence of *bla*$_{SHV}$ gene in raw milk samples from Indian States.[29] However, another study from Anand, Gujarat, India reported 14.7% of isolates were possessing *bla*$_{SHV}$ gene in raw milk samples.[33]

Risk factors for occurrence of ESBL

Based on the results of the statistical analysis ($\chi 2$ test, $P < .01$), 7 variables have been identified to be associated with the presence of ESBL in dairy farms (Table 2). These variables are housing system, milking method, hygienic condition of farm, practice of washing of hands of milkmen as well as udder of animal and water sanitation at farm level. The significant variations in the populations and study methodology across different countries render it extremely challenging to compare the prevalence and risk factors for ESBL production in a reliable manner. Antimicrobial use and subsequent resistance on dairy farms is affected by various management factors

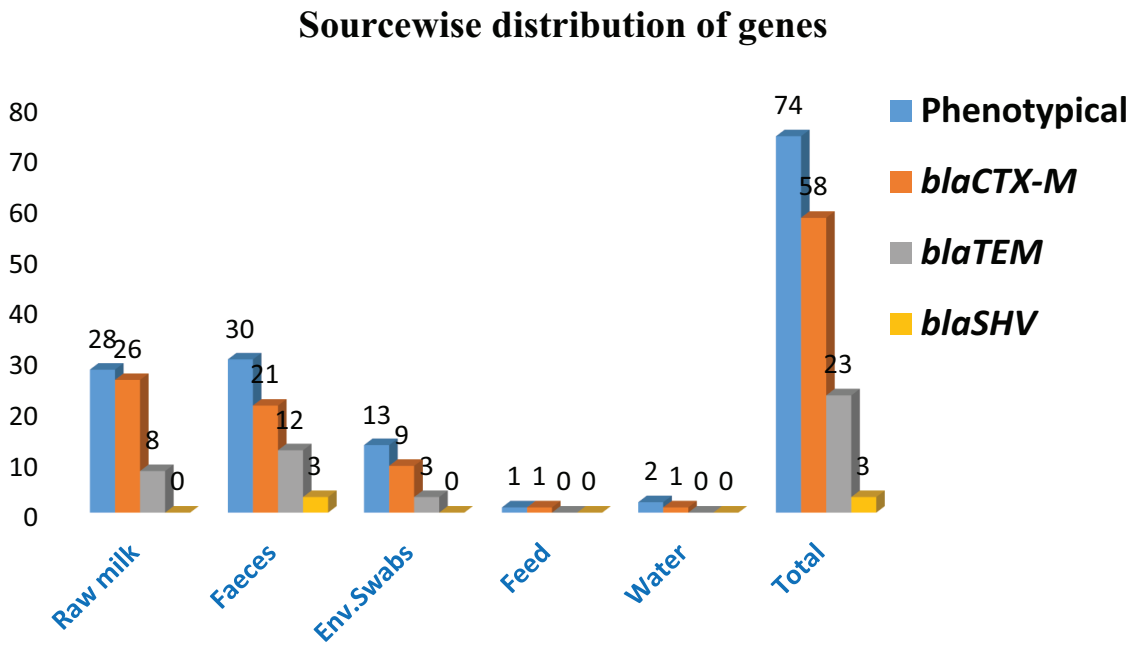

Figure 9. Source wise distribution of β-lactamase genes of ESBL *E. coli*.

Table 2. Risk factor analysis.

VARIABLE	CATEGORY	NO. OF ESBL POSITIVE FARMS	$\chi 2$	*P*-VALUE
Education	Illiterate	06	4.4	.2214
	Matriculate	07		
	Higher secondary	01		
	Graduation	06		
Housing system	Loose system	02	9.7	.0078*
	Conventional barn system	05		
	Semi-intensive system	13		
Milking method	Machine milking	02	24.1	<.0001*
	Full hand milking	17		
	Fist method	01		
Practice of washing of teats before milking	Yes	20	20.0	<.0001*
	No	00		
Practice of washing of teats with $KMnO_4$ after milking	Yes	02	12.8	.0003*
	No	18		
Frequency of animals bathing	Nil	07	3.6	.3080
	Once a day	07		
	Twice a day	04		
	Twice a week	02		
Practice of washing of hands of milkmen before milking	Yes	16	7.2	.0073*
	No	04		
Hygienic condition of farm	Good	14	12.4	.0020*
	Average	02		
	Poor	04		
Source of water supply	Borewell	09	1.3	.522
	Canal	05		
	Domestic supply	06		
Water sanitation at farm level before use	Yes	00	20.0	<.0001*
	No	20		
Therapeutic management	Self/unqualified	12	1.2	.7530
	Diploma in veterinary science	04		
	Veterinary surgeons	04		
Class of antibiotic being used in any therapy	Fluoroquinolone	07	2.8	.4235
	Penicillin	06		
	Cephalosporins	05		
	Aminoglycodides	02		

*$P < .01$

including average herd age, disease outbreaks, cleanliness, the application of teat sealants and farmer's attitudes about antimicrobial stewardship. The housing system of dairy farms can be correlated with the number of animals affected by crowdedness, which in turn leads to easy transmission of resistance genes through different routes. Intensive farming system especially indoor housing can lead to diseases and its associated antibiotic treatment might further leads to AMR.[34] In agreement with our study, other researchers also emphasized the importance of farm hygiene as risk factor for the prevalence of ESBL in dairy farms.[35] Source of drinking water was not found as a significant risk factor associated with the occurrence of ESBL *E. coli* in this study. It was similar to the findings of other studies.[36] Variables related to infrastructure and cleanliness of dairy farm is significantly associated with elevated chance of carrying ESBL as per the study conducted in Israel.[37] They also noticed an elevated carriage of ESBL according to the number of weekly visits of veterinarians in the farm for treatment. In another study, increased use of antibiotics especially cephalosporins are reported as a risk factor for ESBL producing *Enterobacteriaceae* on dairy farms,[36,38] however they were not find hygienic condition as a significant factor.[36] However, there are research study, which couldn't find any correlation[16] between the use of different types of antimicrobials including cephalosporins and ESBL herd status as that of us.

Conclusions

Bacteria with plasmids harbouring antimicrobial resistance genes are a concern for both humans and animals. ESBL producing *E. coli* have become a global health problem in recent years due to their broad diffusion. In this investigation, ESBL producing *E. coli* was present in all the dairy farms included in the current study. However, the percent positivity of the isolation varied in different types of samples. The most common β-lactamase encoding gene from dairy farm environments of Haryana was found to be $bla_{\text{CTX-M}}$. The disseminaton of ESBL *E. coli* within the ecosystem is of serious concern with regard to One Health approach. A holistic multidisciplinary One Health approach involving human, animal and environmental health is required for addressing the issue of antimicrobial resistance.

Author Contributions

Kamboj S carried out the experiment, **Manoj J** designed and supervised the work and drafted the manuscript, **Kaur J** helped in the laboratory works, **Singh M** contributed to the planning of the work and **Chhabra R** was in-charge of overall direction.

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