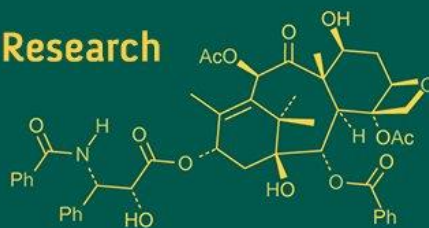


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Prevalence and multidrug resistance profiles of *Escherichia coli* isolated from raw bovine milk

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Abstract

Mastitis is a major disease of dairy animals characterized by severe damage to the lactiferous ducts and mammary alveoli, edema, and infiltration of PMN cells, resulting in partial or complete loss of milk production. *Escherichia coli* is one of the most important environmental pathogens that causes clinical mastitis. This study aimed to investigate the prevalence of mastitis caused by *E. coli*, the major environmental pathogen. The dairy cows were screened for the presence of mastitis by physical examination of the mammary gland and the California mastitis test in order to rule out the status of intramammary infection. The *E. coli* was isolated from the raw cow milk samples collected from 180 cows from clinical cases of mastitis presented at RVP, cattle and buffalo farm, ICAR-IVRI, Bareilly. *E. coli* was isolated on various selective agar media. Bacterial genomic DNA extraction was extracted and molecular detection was done by PCR. The overall prevalence of *E. coli* was 34.44%. Out of the total mastitis positive samples, 37.10% were clinical, and 62.90% were subclinical. The *E. coli* isolates were tested for extended-spectrum β -lactamase production by the combined disc method, and the prevalence of ESBL-producing *E. coli* was found to be 23.53% of the total *E. coli* isolates. The *E. coli* isolates were tested for their susceptibility against 17 antibiotics by the disk diffusion method. Antibigram analysis revealed the highest resistance towards Ampicillin, Amoxicillin/clavulanic acid, and clindamycin (100%), followed by Enrofloxacin, Cefoperazone, Cefepime, Ceftazidime (85.7%), Tetracycline (71.4%), and Ceftriaxone (57.2%). The high prevalence of multidrug-resistant *E. coli* isolates underscores a critical public health risk for consumers, necessitating rigorous surveillance and monitoring programs to mitigate the spread of antimicrobial resistance within the dairy industry.

Keywords: *Escherichia coli*, bovine, ESBL-producing *E. coli*, mastitis

1. Introduction

As illustrated in a 100-year review by Ruegg in the Journal of Dairy Science, mastitis is an inflammatory reaction of the mammary gland, provoked by invading pathogens and their rapid multiplication inside the mammary gland. It remained a major disease of the dairy animals (Stevens *et al.*, 2016; Ruegg, 2017) ^[36]. It is characterized by severe damage to the lactiferous ducts and mammary alveoli, edema, and infiltration of PMN cells, resulting in partial or complete loss of milk production (Lai *et al.*, 2017) ^[18]. It is a serious issue for animal welfare as it causes severe discomfort, pain, a change in behaviour, and hampers the well-being of the affected livestock. Moreover, it also affects human health because of its zoonotic association. It is counted amongst the costliest diseases of milch animals, as it accounts 38% of the overall cost attributed by the major production diseases of dairy animals (Wenz *et al.*, 2006) ^[42]. Despite extensive studies and advances in understanding the pathogenesis of mastitis, it has remained a major concern in the dairy industry worldwide. Among the various etiological agents of mastitis, *Escherichia coli* is one of the most important environmental pathogens that causes clinical mastitis (Blum 2014) ^[3]. Approximately 80 percent of the intramammary infections induced by *E. coli* result in clinical mastitis, and 8-10 percent are per-acute in nature (Verbeke *et al.* 2014) ^[39]. Once established, it did not respond well to the conventional antimicrobial therapy. Endotoxins produced by the bacteria destroy the cell membrane and damage the mammary tissue. Amongst the reported cases of mastitis caused by *E. coli*, a large proportion showed moderate clinical signs. However, around 10% of the cases result in severe clinical signs and even lead to the death of the cow. Bovine mastitis Caused by *E. coli* remained a topic of

concern in many countries despite considerable improvements in management and housing practices. Currently, broad-spectrum antibiotics are being used (systemically or intramammary) in India and other countries for treatment and control of *E. coli* mastitis. In the field, the cases of clinical mastitis are being treated based on the symptoms or manifestations of inflammation and not etiology. Various studies over the past 10 years concluded that *E. coli* mastitis is difficult to treat because of its omnipresent nature, easy spread, and rapidly developing antibiotic resistance (Dahmen *et al.*, 2013) [11]. Moreover, many recent studies have reported the emergence of highly multidrug-resistant strains of *E. coli* isolated from dairy animals from many countries (Seni *et al.*, 2016) [32]. Cost of antibiotics, emergence of drug resistance, and risk of drug residues in milk and meat have pressured the dairy industry to minimize the overall use of antibiotics.

2. Materials and Methods

2.1 Experimental animals

The study was conducted on 180 lactating cows presented at RVP and cattle and buffalo farm ICAR-IVRI, Izatnagar, Bareilly. The lactating cows were screened for the presence of mastitis by physical examination of the mammary gland as per the methods given by Schalm *et al.* (1971) [31] and California mastitis test (CMT) in order to rule out the status of intramammary infection. The milk samples showing positive results, specifically showing score 3(++) or score 4 (+++) were collected for bacterial isolation.

2.2 Collection of milk samples

The samples were collected as per the guidelines given by the National Mastitis Council.

2.3 Isolation and identification of *E. coli*

Milk samples were inoculated in 1 ml MacConkey broth and incubated at 37 °C for 24 hr (Cruickshank, 1962) [10]. A loopful from positive tubes was streaked on MacConkey agar and incubated at 37 °C for 24 hr; pink colonies indicated lactose fermenters. Three to five colonies were transferred to LB broth and incubated overnight, then streaked on EMB agar (Cruickshank *et al.*, 1975) [9]. Colonies showing a metallic sheen on EMB confirmed *E. coli*, which were then subjected to biochemical tests. Approximately 10 µl of the collected sample was inoculated in 1 ml of MacConkey broth. Gram's staining was done for the identification of Gram-negative bacteria. The biochemical characterization of *E. coli* was performed by using HiMotilityTM and HiIMViCTM Biochemical Kit.

2.4 Molecular detection of *E. coli*

Isolation of genomic DNA from the isolated *e. coli* samples

Bacterial genomic DNA was extracted as per the protocol described by Reischl *et al.* (2002) [44] with a few modifications. Single *E. coli* colonies from EMB agar were cultured in LB broth for 24 hours at 37 °C under constant agitation (150 rpm). Aliquots of 1 ml were harvested by centrifugation at 8500 rpm for 5 min, followed by multiple washes of the resultant pellet with nuclease-free water (NFW). Genomic DNA was extracted via thermal lysis in a boiling water bath for 15 minutes, followed by rapid chilling on ice and a final centrifugation to recover the DNA-rich supernatant. The crude extract was subsequently stored at -

20 °C for use as a template in downstream amplification reactions. DNA concentration and purity were assessed spectrophotometrically by loading 1 µl of the sample onto the pedestal of a NanoDrop (or micro-volume spectrophotometer).

2.5 PCR protocol

Species-specific primer sequence as given by Riffon *et al.* (2001) [27] was used for amplification of 16 S rRNA gene of the *E. coli* isolates. The details of the primer used in the study are given in Table 1. The PCR reaction mixture composition and cycling parameters have been given in Tables 2 and 3, respectively. The amplified DNA products were visualized by electrophoresis of 10 µl of PCR product in 1% agarose gel containing ethidium bromide (0.5 µg/ml).

2.6 Agarose gel electrophoresis

The quality of the DNA samples thus extracted was analysed by means of Agarose gel electrophoresis, and the gel was examined in the gel documentation system under UV illumination. The PCR product was determined by comparing with a standard molecular weight DNA ladder and was photographed by the gel documentation system.

2.7 Detection of ESBL producing bacteria

The isolates were tested for Extended Spectrum β-lactamase production by combined disc method as described by CLSI, 2015. In this test, two antibiotics (ceftazidime (30µg) and cefotaxime (30µg)) were used simultaneously, one with 3rd generation antibiotic alone and in another, a combination of the same antibiotic with clavulanic acid. The objective is to see the inhibitory effect of clavulanic acid against beta-lactamase enzyme produced by the isolates.

2.8 Antibiotic sensitivity test

The antimicrobial sensitivity profiles of *E. coli* were determined by disc diffusion method (CLSI, 2019). A total of 17 antimicrobial agents (Ceftriaxone, Gentamicin, Amoxicillin/clavulanic acid, Ampicillin, Co-trimoxazole, Colistin, Piperacillin/Tazobactam, Enrofloxacin, Tetracyclin, Kenamycin, Imipenem, Cefoperazone, Ciprofloxacin, Ceftazidime, Cefepime, Streptomycin and Clindamycin). The antibiotics selected were as per CLSI, 2019.

3. Results

3.1 Screening of lactating cattle

The screening of the lactating cows was done during the period of October-January (winter). A total of 180 lactating cows were screened for mastitis on the basis of CMT and physical examination of the udder and milk. The screened cows were in different lactation stages, housed in a semi-intensive system, and both hand and machine milking methods were used for milking. Out of 180 screened cows, 62 (34.44%) were found positive for mastitis. Physical examination of the udder revealed signs of clinical mastitis, like swelling, redness, hardness, and pain in the affected quarter/udder (Figure 1), and physical examination of milk samples from individual quarters revealed discoloration of milk, the presence of blood, clots, and flakes (Figure 2). Out of the total 62 mastitis positive samples, 37.10% (23/62) were found positive for clinical mastitis, and remaining 62.90% were positive for subclinical mastitis. The result is depicted in Table 4.

The samples with CMT point score 0 were considered as negative, samples with CMT score >1 were considered to have mild to moderate infection and CMT score of > 3 considered to have severe intramammary infection. The samples with CMT score ≥ 1 were collected in sterilized milk collection tubes and processed for bacterial isolation.

3.2 Bacterial isolation

The collected positive samples were inoculated in MacConkey broth. After 24 hours of incubation, 29 out of 62 samples (46.77%) turned yellow, indicating lactose fermentation, whereas no colour change was observed in the remaining 33 (61.53%) samples, indicating non-lactose fermentation (Figure 3). Gram staining of the positive samples revealed gram-negative rod-shaped bacteria in 40% of the samples (Figure 4). On MacConkey agar after overnight incubation, characteristic circular, flat, moist, smooth rose-pink colonies in 19 out of 29 samples and mucoid pink colonies in 10 samples were observed (Figure 5). On EMB agar, characteristic flat, dark colonies with metallic sheen (Figure 6) were observed in 17 (27.42%) isolates, indicating *E. coli*. The Isolates showing a metallic sheen on EMB agar were inoculated into the biochemical test kits and incubated overnight. All the 17 isolates showed characteristic colour change after overnight incubation and on addition of the reagent (Figure 7). Results were interpreted as per the interpretation chart provided with the kit. Results revealed that all the isolates were motile, positive for the indole, methyl red, and nitrate reduction tests, and negative for Voges-Proskauer and Citrate utilization tests. All the isolates were found to ferment glucose, lactose, sorbitol, arabinose, mannitol, and rhamnose, whereas variable results were found for sucrose fermentation.

3.3 Molecular detection of *E. coli*

Bacterial genomic DNA was isolated from the samples (n=17) showing a characteristic metallic sheen on EMB. Nanodrop spectrophotometer reading of the isolated DNA samples revealed DNA concentration ranged from 51ng/μl to 287ng/μl and purity ranged from 1.4-1.8 ($A_{260/280}$), indicating that the samples were almost free from contamination (protein/RNA). DNA isolation was repeated for the samples having less than 1.5 purity and less than 50 ng/μl DNA concentration, and samples having very low DNA concentration were discarded. PCR application of the 16S rRNA gene was done using the designed primers as given in Table 1. The PCR amplified products were run on 1.5% agarose gel. The gel was examined under a gel documentation system. A single band of approximately 232 bp in size in all 17 samples (Figure 8) was observed. No band was observed in the negative control (non-template) well.

3.4 Detection of ESBL-producing bacteria

After overnight incubation, a difference of 50% or 5mm or more between the zone of inhibition of the discs containing the antibiotic along with clavulanic acid and the antibiotic disc containing the antibiotic alone was observed in 4 out of 17 (23.53%) *E. coli* isolates, indicating the presence of ESBL-producing *E. coli* (Figure 9).

3.5 Antibiotic susceptibility testing of *E. coli* isolate

All the confirmed *E. coli* isolates (n=17) were tested for *in vitro* antibiotic sensitivity towards different antibiotics

(n=17). These antibiotics belonged to the 7 groups of antibiotics viz. aminoglycosides, tetracycline, penicillin, beta-lactam, fluoroquinolones, cephalosporins, and sulphonamide. Results were interpreted as per the manufacturer's instructions (HiMedia) (Figure 10). The highest resistance was found towards antibiotics Ampicillin, Amoxicillin/clavulanic acid and clindamycin (100%) followed by Enoxofloxacin, Cefoperazone, Cefepime, Ceftazidime (85.7%), Tetracyclin (71.4%), Ceftriaxone (57.2%) The highest sensitivity was attributed towards Gentamicin and Imipenem (100%) followed by Kanamycin (85.7%), Piperacillin/Tazobactam, Co-trimoxazole, Colistin and Streptomycin (71.4%). Additionally, 57.2% of the isolates showed intermediate susceptibility towards ciprofloxacin. The results are depicted in Table 5. According to the results of ABST *E. coli* isolate (KJ933691) showed least sensitivity against Amoxicillin/clavulanic acid and also it is the most commonly used antibiotic in the field hence, Amoxicillin/clavulanic acid was chosen for experimental trial.

4. Discussion

Screening of lactating cattle and isolation of *E. coli*

In the present study the overall prevalence of mastitis on the basis of CMT and physical examination of the udder and milk was found to be 34.44%. Out of the total mastitis positive samples, 37.10% were clinical and 62.90% were subclinical. For isolation of *E. coli* the samples were streaked on MacConkey agar and EMB. The positive samples showed characteristic circular, flat, moist, smooth rose-pink colonies on MacConkey agar and metallic sheen on EMB agar, indicating presence of *E. coli*. The samples were further confirmed by gram staining, biochemical test and PCR. Gram staining revealed gram-negative rod-shaped bacteria, biochemical test revealed typical ++- IMViC pattern, and PCR revealed a single band of approximately 232bp in size, thus confirming the isolated bacteria is *E. coli*. Similar findings were reported by Riffon *et al.* (2001) [27], Mohanty *et al.* (2013) [22], Singh *et al.* (2018) [34] and Sangeetha *et al.* (2020) [30]. The prevalence of mastitis caused by *E. coli* was found to be 27.42%. Comparison of these findings with the previous reports from the same dairy farm concluded that the overall prevalence of mastitis obtained in the present study (34.44%) is lower than the previous findings i.e. 43.69%, 47.7% and 48% as recorded by Shete (2012) [33], Radhika (2016) and Sangeetha (2018) [29], respectively. However, the prevalence of mastitis caused by *E. coli* found in this study (27.42%) is higher than the previous studies from the same farm viz. 10% and 13.33% as reported by Snehal (2006) [35] and Shete (2012) [33], respectively. The high prevalence in this study may be attributed by the poor hygienic conditions because *E. coli* is the part of normal intestinal flora in bovine and can contaminate the environment. During the puerperal period when the cow's immunity is compromised, they may get opportunity to enter and multiply in the mammary gland of the cow and cause mastitis (Radostits *et al.*, 2007) [16]. The previous studies, from different farms reported variable prevalence for *E. coli* mastitis. The studies published by Balakrishnan *et al.* (2004) [2], Breen *et al.* (2009) [5], Vasquez *et al.* (2019) [38] and Sangeetha *et al.* (2020) [30] reported a prevalence of 27.5%, 26.7%, 23% and 25.7%, respectively which is similar to the findings of the present study. However, a higher prevalence than the present study

is reported by Bradley and Green (2001)^[4], Mohsen and Dapgh (2007)^[23] and Paramesh *et al.* (2018)^[24] i.e. 34.7%, 39.9% and 42%, respectively. Some other studies reported lower prevalence than the present finding viz. 20.0% (Sumathi *et al.*, 2008)^[37], 21% (Mohanty *et al.*, 2013)^[22], 20.10% (Charaya *et al.*, 2014)^[6] and 17.19% (Singh *et al.*, 2018)^[34]. The *E. coli* isolates were also examined for the presence of ESBL and the prevalence of ESBL producing *E. coli* was found to be 23.53% of the total *E. coli* isolates. The findings were in correlation with results reported by Ali *et al.* (2017)^[1] and Feng *et al.* (2018)^[14], who found the overall prevalence to be 25.41% and 22.96, respectively. However, the prevalence found in this study is higher than reported by Kar *et al.* (2015)^[17], Freitag *et al.* (2017)^[15] and Eisenberger *et al.* (2018)^[12]. The widespread use of β -lactam group of therapeutic agents in curing and preventing cases of bovine mastitis may result to this phenomenon. The presence of ESBL in mastitic milk underlines the value of udder hygiene. It is important to carry out proper and modified drug treatment, strictly following therapeutic protocols, in order to avoid pharmacokinetic conditions which, encourage the production of strains carrying antibiotic resistant genes (Locatelli *et al.*, 2009)^[19].

Antibiotic susceptibility testing of *E. coli* isolates

All the confirmed *E. coli* isolates (n=17) were tested for *in vitro* antibiotic sensitivity towards different antibiotics (n=17). In the present study, the highest sensitivity was attributed towards Gentamicin and Imipenem (100%), followed by Kenamycin (85.7%), Piperacillin/Tazobactam, Co-trimoxazole, Colistin and Streptomycin (71.4%). The resistance pattern revealed, highest resistance against Ampicillin, Amoxicillin/clavulanic acid and clindamycin (100%) followed by Enoxofloxacin, Cefoperazone, Cefepime, Ceftazidime (85.7%), Tetracyclin (71.4%), and Ceftriaxone (57.2%). Approximately 57.2% of the isolates showed intermediate susceptibility towards ciprofloxacin. Present study is in agreement with the findings of Preethirani *et al.* (2015)^[15], who tested antibiotic resistance of *E. coli* strain isolated from mastitis cases in buffalo. Their study reported 100% resistance against Amoxycillin, Amikacin, Ampicillin, Ceftriaxone/ sulbactam, Cefotaxime, which is in corroboration to the present study. Similarly, Sumathi *et al.* (2008)^[37], Virpari *et al.* (2013)^[41], Charaya *et al.* (2014)^[6] and Verma *et al.* (2018)^[40] reported the highest sensitivity of *E. coli* isolates against Gentamicin, which strongly supports the findings of this study. However, the percentage of resistance towards other antibiotics was lower than the present study. Zhang *et al.* (2018)^[43] in their study reported 8.86%, 11.39%, 25.32%, and 25.33% resistance against Enoxofloxacin, Amoxycillin, Ampicillin, and tetracycline, which is much lower than the findings of the present study. Moges *et al.* (2011)^[21] reported the highest sensitivity towards clindamycin, which is not in corroboration with the present study, as 100% resistance was observed in this study against *E. coli* isolates from bovine mastitis. In the present study, a lower resistance rate was observed against Co-trimoxazole, which is supported by the findings of Fazel *et al.* (2019)^[13] (18.3%). But the % resistance against Enoxofloxacin (22.5%) reported by Fazel *et al.* (2019)^[13] was lower than the present study (85.7%). Antibiotic resistance is an important public health issue in medical as well as veterinary science. Extensive use of these antibiotics in the studied dairy farm for treating cases of

clinical mastitis may be a reason behind the high antimicrobial resistance in this study. Mahantesh and Basappa (2011)^[20] and Jeykumar *et al.* (2013)^[16] from their studies also concluded that increased resistance against the commonly used antibiotics can be due to their injudicious use as well as negligence in the bacteriological analysis of the mastitis suspected milk samples, resulting in increased treatment cost as well as period. While species, geographic, and management differences result in differences in the findings of the antibiogram study, the choice of antibiotics used by various workers often contributes to misleading correlations between the studies concerning the susceptibility of mastitis-causing pathogens towards antibiotics.

Conclusion

The present study highlights the significant prevalence of *Escherichia coli* in bovine mastitic milk and emphasizes its growing antimicrobial resistance pattern in dairy animals. A considerable proportion of the isolates were obtained from subclinical mastitis cases, indicating that apparently healthy animals may silently contribute to the persistence and spread of infection within dairy herds. The detection of ESBL-producing *E. coli* and the high level of resistance against commonly used antibiotics further reflect the increasing therapeutic challenges associated with mastitis management. The widespread multidrug resistance observed among the isolates is a matter of serious concern for both veterinary and public health sectors, as resistant bacteria from raw milk may enter the food chain and pose a potential zoonotic risk to consumers. These findings underline the urgent need for regular microbiological screening, judicious use of antimicrobials, and implementation of effective hygiene and herd management practices at dairy farms. Overall, this study provides important baseline information on the prevalence and antimicrobial resistance characteristics of mastitis-associated *E. coli* in dairy cattle and supports the need for continuous surveillance programs to control antimicrobial resistance and improve milk safety and animal health.

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Competing interest's disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or conflict with the subject matter or materials discussed in the manuscript.

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