

Occurrence and characterization of multidrug-resistant extended spectrum β -lactamase producing *Escherichia coli* from bovine mastitis of Haryana

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ABSTRACT

A study was carried out to analyze 500 bovine milk samples collected from Haryana, India and 37% samples were found to be associated with *E. coli* as a causative agent for mastitis. Out of it, 53 (10.6%) isolates were found to be positive for ESBL production by Double-Disk Synergy test and Epsilon test. However, the ESBL-encoding gene *bla*_{CTX-M} was detected in the 51 (10.2%) isolates. The antibiotic susceptibility test revealed 13.72% of the isolates were multidrug resistant, with MAR index varying from 0.06 to 0.46. The maximum sensitivity was observed towards gentamicin (90.19%) followed by chloramphenicol (78.4%). Fluoroquinolones antibiotics were found to be resistant between 84.31% to 76.47%. The resistance profile underscores the importance of antibiotic stewardship and the need for ongoing surveillance using one health approach to guide therapy and manage the spread of resistant bacteria.

Keywords: Antibiotic resistance, DDST, ESBL, Gram negative, MAR index

Mastitis is the most common disease that affects dairy animal's productivity. Dairy farmers in low and middle-income countries including India suffer financial losses as a result of mastitis in terms of decreased production as well as treatment and prevention costs (Sasidhar *et al.* 2002, Sinha *et al.* 2014, Jamali *et al.* 2018, Cobirka *et al.* 2020). The spread of harmful microorganisms and their toxins through the milk and dairy products has an impact on public health (Shaheen *et al.* 2015, Argaw 2016). The global problem of antimicrobial resistance has an impact on animal and human health. Efforts in the field of welfare of animals has been enabled by impacts about the development and dissemination of antibiotic resistance (Naranjo-Lucena and Slowey 2023). Antibiotic resistance among mastitis causing and foodborne pathogens is well reported from India (Das *et al.* 2017, Yadav *et al.* 2018, Manoj and Singh 2020, Dey *et al.* 2023). The OIE advises monitoring antibiotic resistance in animals while WHO urges prudent and sensible use of antibiotics in the community as a whole (Doughnon *et al.* 2020). Due to the extensive use of antibiotics in the treatment and prevention of mastitis, there is a chance that milk and its products will contain antibiotic residues and multidrug-resistant bacteria that could be harmful to human health (Deb *et al.* 2023).

Escherichia coli was recognized as the environmental mastitis pathogens (Belay *et al.* 2022, Goulart and Mellata 2022). Beta lactam antibiotics are widely used

for the treatment of bovine mastitis caused by Gram negative bacteria. Extended-spectrum beta lactamases (ESBL) enzymes provide resistance to most beta lactam antibiotics used in human as well as veterinary profession medicines especially cephalosporins. Enterobacteriaceae containing genes encoding ESBL, AmpC-lactamases or carbapenemases have emerged and spread quickly in recent years. Enterobacteriaceae acquire ESBL by several mobile genetic elements mediated by horizontal gene transfer (Deb *et al.* 2023, Gelalcha *et al.* 2023). *E. coli* that produces ESBL has also been detected in raw milk with or without mastitis symptoms (Ansharieta *et al.* 2021). ESBL producing Gram negative isolates can be transmitted between humans and animals by various routes like direct contact and through food chain via raw milk and its products (Batabyal *et al.* 2018, Tenhagen *et al.* 2020, Dey *et al.* 2023). This study was designed to know about the presence of ESBL producing *E. coli* from bovine mastitis cases of Haryana, India. The characterization and antimicrobial susceptibility pattern of ESBL *E. coli* were also analyzed to know the resistance profile.

MATERIALS AND METHODS

Samples: The milk samples routinely submitted to the College Central Laboratory, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana were used in this study. These samples were from the bovines of Haryana and examined for its appearance (Clinical mastitis) and CMT test (Subclinical cases). The mastitis positive samples were subjected to the cultural examination and antibiotic sensitivity testing. Further, the details of

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each case were recorded from the owners regarding the milk yield, stage of lactation, abnormalities of udder/teats, fibrosis, inflammatory swelling and nature of case (acute, sub-acute or chronic) and the treatment undergone if any.

Bacterial isolation and identification: A loopful of milk (~10 µL) from each sample was streaked onto MacConkey agar (HiMedia laboratories Pvt. Ltd, Mumbai, India) and incubated at 37°C for 18-24 h. The lactose fermenting pink to red colonies were further streaked onto Eosin Methylene Blue agar (Hi Media, Mumbai) and incubated overnight at 37°C. The dark centered 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, oxidase and nitrate reduction tests (Quinn *et al.* 2011). Confirmation of *E. coli* isolates was carried out by detection of *uidA* gene by PCR (Behera *et al.* 2023).

Phenotypic detection of ESBL production: The *E. coli* isolates were checked for extended spectrum beta-lactamases production by chromogenic agar plate method. The *E. coli* isolates were streaked onto the ESBL agar base media (Hi Media, India) and incubated for 24 h at 37°C. The colonies with purple colour were tentatively identified as ESBL producer.

The ESBL producing isolates were further verified by Double-Disk Synergy Test (DDST) using 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 Muller Hinton agar 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 (CLSI 2020).

The isolates were also subjected to Minimum Inhibitory Concentration (MIC) test/Epsilon test using MIC strips (EM132, HiMedia laboratories Ltd., Mumbai). The MIC values were noted at the point where ellipse of inhibition zone intersects the MIC scale on the strip as per the manufacturer's instructions.

Molecular detection of ESBL encoding genes: Phenotypically positive ESBL *E. coli* isolates were checked for the presence of ESBL encoding gene *bla*_{CTX-M} (Deb *et al.* 2023). The DNA from the *E. coli* isolates was extracted using QIAGEN mini kit. The primers were synthesized from Integrated DNA Technologies with oligonucleotide sequences of 5'-CGCGGTGCTGAAGAAAAGTG-3' and 3'-GCCGGTTTTATCCCCACAA-5' for forward and reverse primers, respectively. Briefly, the reaction mixture was prepared using 12.5 µL of master mix (Promega, USA), 1 µL of each primer (10 µM), 2 µL DNA template

and nuclease free water to make the final volume up to 25 µL. The polymerase chain reaction was carried out with an initial denaturation at 95°C for 5 min, followed by 30 cycles each of denaturation at 95°C for 30s, annealing at 60°C for 30 s and extension at 72°C for 30 s with final elongation at 72°C for 5 min. The amplified product was resolved on electrophoresis gel (1.5%) containing ethidium bromide at a concentration of 0.5 µg/mL and the gel was run at 75 V for 1.5 h 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.

In vitro antimicrobials sensitivity testing: The bacterial isolates obtained were tested for their antibiotic susceptibility pattern using the Kirby-Bauer disc diffusion method according to CLSI guidelines (CLSI 2020). A total of 15 different antibiotics from six different classes were used, which included amoxicillin (10 µg), ampicillin (10 µg), amikacin (30 µg), chloramphenicol (30 µg), cefoperazone (75 µg), ceftriaxone (30 µg), enrofloxacin (10 µg), gentamicin (10 µg), kanamycin (30 µg), levofloxacin (5 µg), moxifloxacin (5 µg), neomycin (30 µg), oxytetracycline (30 µg), penicillin (10 units) and streptomycin (10 µg). The isolates were classified as resistant, intermediate or sensitive towards the tested antibiotics based on the zone of inhibition developed as per manufacturer's quality control instructions (Hi Media laboratories Pvt. Ltd, Mumbai, India). Multiple antibiotic resistance (MAR) index was determined by using the formula:

$$\text{MAR} = a/b$$

where 'a' represents the number of antibiotics to which the test isolate shown resistance and 'b' represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility (Krumperman 1983).

RESULTS AND DISCUSSION

Out of 500 bovine milk samples analyzed, 185 (37%) samples showed *E. coli* as causative agent for mastitis. All these isolates were given typical phenotypical characteristics of *E. coli*. These isolates were lactose fermenting on MLA, showing typical metallic sheen on EMB agar and were Gram negative having pink rods upon Gram staining. All the presumptive *E. coli* isolates were positive for indole, methyl red, catalase and nitrate reduction tests, while they were negative for Voges-Proskauer, oxidase test and citrate utilization. The increased incidence of coliform mastitis could be attributed to unhygienic environment as well as improper milking practices which elevate the risk of environmental mastitis (Shaheen *et al.* 2015, Yadav *et al.* 2023). The environmental factors like hygiene level of animal, udder and milking machine, milking practices, milker's hygiene and housing system plays crucial role in the occurrence of environmental mastitis.

A total of 56 isolates (11.2%) showed purple-coloured

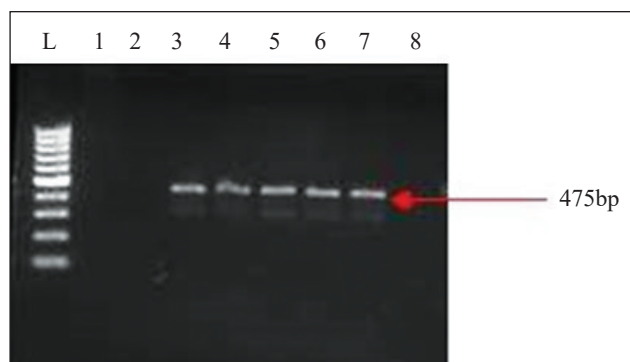


Fig. 1. PCR amplified product of *bla*_{CTX-M} gene [L: 100 bp ladder, 1-6 samples; 7: Positive control; 8: Negative control].

colonies on chromogenic agar and these were considered as ESBL positive, presumptively. These isolates were further confirmed by Double-Disk Synergy Test and 53 isolates (10.6%) were found to be positive for ESBL production. All the ESBL positive strains were shown ellipse of inhibition, only at the end where the clavulanic acid was present in the MIC strip. This further confirmed the production of ESBL by the isolates (Rawat and Nair 2010). The MIC values of these isolates were noted and were ranged between 0.25 to 0.048. Seventeen isolates (32.08%) were given MIC reading at 0.094, while 13 isolates (24.53%) showed ellipse of inhibition at 0.064. There are few reports in India about the prevalence of ESBL *E. coli* in mastitis milk from bovines (Bandyopadhyay *et al.* 2015, Paghdar *et al.* 2020, Yadav *et al.* 2023). The results of current study were comparable with other reports of ESBL producers from India. A prevalence rate of 6.78% was reported for ESBL *E. coli* from buffalo mastitis in Hisar (Yadav *et al.* 2023). An overall prevalence of 9% was reported in animal origin samples for ESBLs from India (Kuralayanapalya *et al.* 2019).

The ESBL-encoding gene *bla*_{CTX-M} was detected in the 51 *E. coli* isolates (10.2%) from bovine mastitis cases

investigated in this study (Fig. 1). In Asian countries, especially the Indian subcontinent, ESBL producers possessing *bla*_{CTX-M} gene were found to be more prevalent having considerable clinical effects (Koovapra *et al.* 2016, Paghdar *et al.* 2020). ESBL genes may transmit between bacterial isolates *via* the exchange of mobile genetic components like plasmids which may carry extra antibiotic resistance genes (Behera *et al.* 2023). Food animals could be a significant source of drug-resistant microorganisms or genes to humans. There are reports of cross-transmission of ESBL genes between animal origin foods and human. These pathogens have been found to be easily transmitted into the human food chain through milk or from environment (Koovapra *et al.* 2016, Ansharieta *et al.* 2021, Gucukoglu *et al.* 2023). Resistance-gene carrying *E. coli* is capable of transmitting its virulence genes across other pathogens with ease (Batabyal *et al.* 2018).

The antibiotic susceptibility test revealed 13.72% of the isolates were multidrug resistant, with resistance to three or more antibiotics from three different antibiotic classes (Ibrahim *et al.* 2016). The MAR index was found to be varying from 0.06 to 0.46 in this study with an alarming impact on public health. MAR index ≥ 0.2 originate from a high-risk source of contamination where several antibiotics are used (Krumperman 1983). The maximum sensitivity was observed towards gentamicin (90.19%) followed by chloramphenicol (78.4%) in our study. These antibiotics inhibit bacterial protein synthesis and suggest that gentamicin would be a good choice for treating infections caused by ESBL *E. coli*. The result of current study is in agreement with the other reports from Haryana. Manoj and Singh (2020) reported maximum sensitivity to chloramphenicol (71.62%) for Gram negative isolates obtained from mastitis cases of Southern Haryana. Yadav *et al.* (2023) reported *E. coli* isolates showed 100% sensitivity to gentamicin in their study. The lowest resistance to this antibiotic seems to be associated with

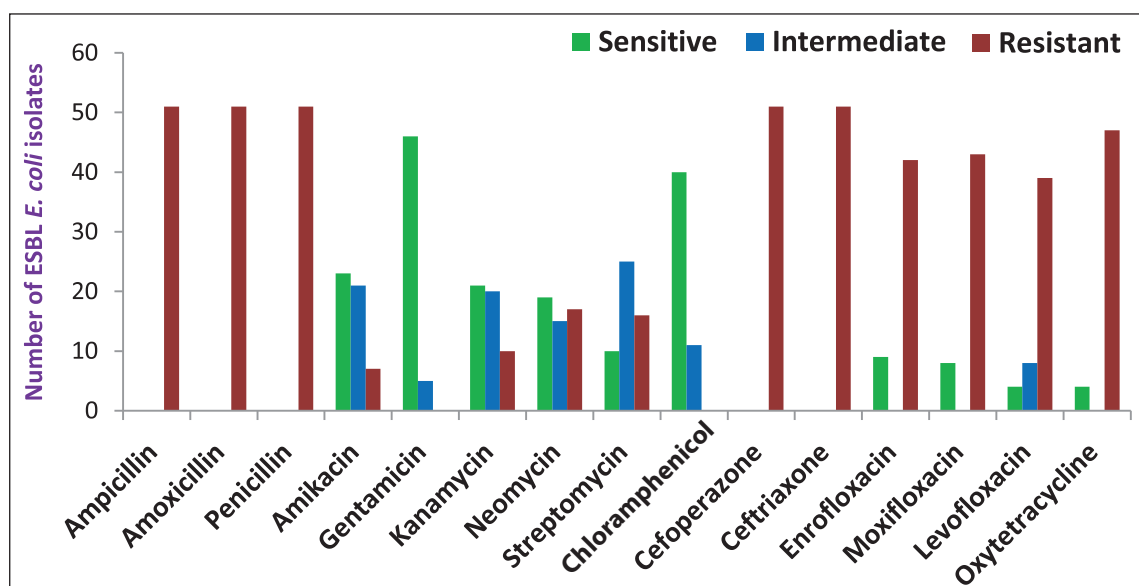


Fig. 2. Antibiotic susceptibility pattern of ESBL *E. coli* isolates.

the restricted usage along with the reduced preference of gentamicin in the treatment of mastitis (Yadav *et al.* 2023).

The tetracycline showed a resistance level of 92.16%, which might be due to efflux pumps or ribosomal protection mechanisms (Chopra and Roberts 2001). The range of resistance to the fluoroquinolones antibiotics was 84.31% to 76.47% (Fig. 2). Resistance to fluoroquinolones can arise through mutations in the target enzymes or efflux mechanisms. Since plasmids encode the majority of ESBLs, the co-resistance to other antibiotic classes was quite frequent (Vasquez *et al.* 2017). Complete resistance was observed for beta-lactam antibiotics, due to the production of beta-lactamases enzymes that break down the antibiotic before it can exert its effect.

The presence of ESBL-producing *E. coli* poses a significant hazard as it can lead to treatment failures. The detection of ESBL producers from bovine milk is worthwhile in view of the possibility that people may inadvertently consume the infected milk and its products leading to asymptomatic colonization that develops into a complex systemic infection. An integrated strategy like One Health is necessary to comprehend and identify the possibility of preventing the spread of the ESBL encoding virulence genes. The resistance profile underscores the importance of antibiotic stewardship and the need for ongoing surveillance to guide therapy and manage the spread of resistant bacteria. Gentamicin appears to be the most effective choice for therapy in this study, with chloramphenicol as a secondary option. Beta-lactam antibiotics are advised to be used in conjunction with enzyme inhibitors such as clavulanic acid for cost effective therapy.

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