Original Article

Detection of antimicrobial resistance genes in extended spectrum beta-lactamase-producing *Escherichia coli* from milk of indigenous Beetal goats of Punjab

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10.22099/IJVR.2023.43480.6365

(Received 12 Apr 2022; revised version 2 Jan 2023; accepted 7 Jan 2023)

Abstract

Background: Antimicrobial resistance (AMR) is a burning issue in the present era. Mastitis in dairy animals is one of the most important causes of huge production loss to dairy farmers. **Aims:** The study aims to find the prevalence, antimicrobial resistance profile, and resistance genes in the extended-spectrum beta-lactamase-producing *Escherichia coli* in mastitic milk. **Methods:** A total of 125 milk samples were collected from Beetal goats suffering from clinical mastitis from different districts of Punjab and processed for bacterial isolation and further identification. The drug resistance profile of ESBL-producing *E. coli* and its associations with molecular markers was analyzed using statistical analysis. **Results:** The prevalence of ESBL-producing *E. coli* in dairy goats of Punjab was recorded as 6.4%. The isolates showed the highest resistance to the beta-lactam group of antibiotics. The resistance percentages of streptomycin, gentamicin, tetracycline, chloramphenicol, clotrimazole, and colistin were 50%, 37.5%, 50%, 25%, 25%, and 50%, respectively. The isolates showed intermediate resistance to imipenem (12.5%) and tetracycline (25%). The ESBL-producing *E. coli* isolates harbored the resistance genes *blaCTXM* (100%), *blaTEM* (62.5%), *blaSHV* (25%), *blaOXA* (37.5%), *tetA* (37.5%), *tetB* (25%), *aadA* (37.5%), *sul1* (25%), *MOXM* (12.5%), *DHAM* (25%), and *blaCMY-2* (50%). Tetracycline and sulphonamide resistances were statistically associated with their respective resistance genes ($P<0.05$). Streptomycin resistance was not statistically associated with the presence of the *aadA* gene ($P>0.05$). The genes *blaIMP* and *blaNDM* were not recorded in any of the isolates. In this study, 12.5% of the isolates showed co-resistance to colistin and carbapenem. **Conclusion:** Antimicrobial resistance is a hot topic and requires immediate attention.

Key words: Antimicrobial resistance, ESBL, Mastitis, Pathogens

Introduction

Mastitis is one of the most common and economically important diseases in the food animal production system, as it is responsible for colossal production loss to dairy farmers. The majority of antibiotics used in the dairy industry are for treating mastitis in dairy herds. Previous works showed that the frequently used antimicrobial groups are penicillins, cephalosporins, tetracycline, chloramphenicol, amino-glycosides, and sulphonamides in the dairy industry. Moreover, the widespread use of antibiotics has resulted in the emergence of resistant bacteria (Sharma *et al*., 2018). Antimicrobial resistance (AMR) is one of the urgent issues in the present era. It occurs when a previously sensitive organism becomes resistant to an antimicrobial drug upon misuse. The organism becomes resistant to the drug action through various means like changing the target site of the drug, pumps for rapid efflux, synthesizing drug degrading enzymes, and altering the membrane permeability. All these actions are mediated through genes that express a different polypeptide not encountered previously, thus making it more efficient to deal with the antimicrobials (Harbottle *et al*., 2006). The problem arises because these genes are seldom self-limiting; instead, they propagate in the progenies and wide varieties of the bacterial population, thus transferring resistance in them, e.g., *blaCTXM* transferred from non-pathogenic *Kluyvera* spp. to the members of the family *Enterobacteriaceae*. WHO listed *Escherichia coli* as the most critical group of bacteria that pose a threat of rapid development of resistance to various antimicrobial groups, thus resulting in deadly infections such as urinary tract infections (UTI), bloodstream infections, and pneumonia. These are generally commensals occurring in the GI tract of

humans and animals. Coliforms are normal inhabitants of the soil and intestine of animals. They multiply in the contaminated bedding materials, polluted water, and soil, thus acting as common environmental pathogens responsible for causing mastitis in dairy animals. These bacteria gain entry into the teat canal due to a lack of hygienic milking practices. A vast population accompanied by urbanization and increased demand for food products has pressured farmers to produce more in less time (Vishnuraj *et al*., 2016). Lack of proper education among farmers regarding antimicrobial resistance has promoted the use and misuse of a myriad of antibiotics in day-to-day life for treating diseases, and promoting growth in animals as prophylaxis (Mutua *et al*., 2020). Antibiotic usage has increased over the past few years. It acts as an artificial selection pressure, promoting the emergence and propagation of resistant pathogens responsible for passing the resistance genes to their progeny. Aminoglycosides, carbapenems, chloramphenicol, and colistin are listed as the drug of last resort. The rapid growth and propagation of multidrug resistance pathogens have highlighted the significance of their investigation in food animals. This study aims to investigate the prevalence, antimicrobial resistance, and resistance genes of ESBL-producing *E. coli* in mastitic goat milk.

Materials and Methods

Sample collection

The study was funded by the Indian Council of Agricultural Research (ICAR). The current study was ethically approved by Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, IAEC Approval No. (497/GO/Re/SL/02/CPCSEA dated 10.11.2016). A total of 125 milk samples were collected from Beetal goats suffering from clinical mastitis from different districts of Punjab. Twenty milk samples from healthy animals were also included in the study. The districts covered were Ludhiana, Ferozepur, and Moga. The animals had clinically affected udders along with altered milk quality. The samples were collected aseptically from both the udders of the animal and transported to the laboratory in an icebox. They were immediately processed for bacterial isolation.

Sample processing, bacterial isolation, and identification

All the samples were initially inoculated into Brain Heart Infusion agar (HiMedia, India) plates and kept for overnight incubation at 37°C. After that, individual colonies were re-streaked on HiCrome Universal Differential media (HiMedia, India). The isolates showing characteristic purple colonies were suspected of *E. coli*. The isolates were inoculated into MacConkey Lactose agar (HiMedia, India) in order to check the lactose fermentation and other colony characteristics. The isolates suspected of being *E. coli* were further streaked on Eosin Methylene Blue agar (HiMedia, India) to check the production of metallic sheen. The bacteria were identified on the basis of cultural, morphological, and biochemical characteristics. All isolates were subjected to various biochemical tests to identify the organism like catalase, oxidase, indole test, Methyl Red test, Vogues Proskauer test, citrate utilization test, urease production, and nitrate reduction.

Bacterial genomic DNA extraction

Genomic DNA was extracted using the hot, cold lysis method in which a few bacterial colonies taken from a pure fresh culture were taken using an inoculation loop and resuspended in 100 µL of nuclease-free water. After that, the suspension was vortexed for 30 s and kept in a dry bath at 100°C for 10 min, and immediately cooled on ice for 15-20 min. After that, the suspension was centrifuged.

Identification of *E. coli* using PCR

E. coli was molecularly detected using forward primer 5´ ATC AAC CGA GAT TCC CCC A 3´ and reverse primer 5´ TCA CTA TCG GTC AGT CAG CAG GAG 3´ as reported (Riffon *et al*., 2001). An amplicon of size around 232 bp was considered as *E. coli.* Gel electrophoresis was carried out using 1.5% molecular grade agarose gel at 80 V for 1 h. The gel was visualized using a UV illuminator (AlphaImager, Innotech).

Phenotypic screening for extended spectrum beta-lactamase production

The *E. coli* isolates were phenotypically screened for ESBL production using the combined disc method. Cefotaxime (CTX, 30 µg) and cefotaxime in combination with clavulanic acid (CEC, 30/10 µg), ceftazidime (CAZ, 30 µg), and ceftazidime in combination with clavulanic acid (CAC, 30/10 µg) discs were used for screening the isolates. A positive result was indicated by an increase in the zone of inhibition by ≥5 mm (synergy effect) around discs with clavulanic acid than without.

Antibiotic sensitivity testing

Antibiotic sensitivity testing was carried out as per CLSI guidelines using the standard disc diffusion method. A total of 17 antibiotics commonly used (based on the history collected from farm owners) were tested, and the resistance pattern was noted down. Antibiotic discs (Himedia, India) used in this study comprised ampicillin-sulbactam (10 mcg), amoxicillin plus clavulanic acid (30 mcg), streptomycin (10 mcg), gentamicin (10 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg), clotrimazole (25 mcg), cefazoline (30 mcg), cefixime (5 mcg), cefoxitin (30 mcg), cefoperazone (75 mcg), ceftriaxone (30 mcg), cefotaxime (30 mcg), ceftazidime (30 mcg), aztreonam (30 mcg), imipenem (10 mcg), and colistin (0.25-16 mcg/ml). Broth microdilution for colistin was performed, and the MIC result was compared with the indicator provided in the kit (HiMICMPK020Colistin) to determine colistin resistance. This was noted as per MIC

values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Molecular detection of antimicrobial resistance genes

The AMR genes targeted were *blaTEM*, *blaCTXM1*, *blaCTXM-3*, *CMY-2*, *blaSHV*, *blaOXA*, *DHAM*, *MOXM*, *blaIMP*, *blaNDM*, *tet A*, *tetB*, *aadA*, and *sul1*. The genes and their association with resistance against the above-stated antibiotics were studied. The primers used for the amplification of the genes are stated in the Supplementary Table 1 (ST1).

Statistical analysis

Statistical analysis was carried out to find whether there is an association between the occurrence of AMR genes and resistance to the antibiotic groups stated using IBM SPSS version 15.0 software. Nonparametric viz. The Chi-square test was performed to find associations. $P<0.05$ results were considered statistically significant.

Results

A total of 22 *E. coli* were isolated from 125 mastitic milk samples. Thus, the prevalence of *E. coli* in caprine mastitis was recorded as 17.6%. The *E. coli* isolates were positive for indole, methyl red, and negative for Voges Proskauer test and citrate utilization test. The *E. coli* isolates were catalase-positive and oxidase-negative. All the isolates formed lactose fermenting pink-coloured colonies on MLA and produced metallic sheen on EMB agar. The isolates were motile, as seen from their growth away from the stab line in sulphide indole motility (SIM) media. Out of 22 *E. coli* isolates, 8 screened positive for extended spectrum beta-lactamase production by Combined disc method. The district wise prevalence was recorded to be highest for Ludhiana, followed by Ferozepur and Moga (Table 1). All the ESBL-producing *E. coli* isolates obtained were multidrug-resistant (MDR), showing resistance to >= three antimicrobial categories tested. The highest resistance was recorded for the beta-lactam group of antibiotics, including extended-spectrum cephalosporins, followed by tetracycline, imipenem, streptomycin, and colistin. The isolates were most sensitive to chloramphenicol and clotrimazole, followed by gentamicin (Fig. 1, Table 2). Statistical analysis revealed a high correlation between AMR genes and resistance conferred ($P<0.05$). The statistical association between AMR genes and antibiotic resistance could be seen for *tet* and *Sul1* genes ($P<0.05$). In contrast, no association was recorded for the presence of the *aadA* gene and streptomycin resistance ($P>0.05$). The ESBL-producing *E. coli* isolates harbored the resistance genes viz *blaCTXM* (100%), *blaTEM* (62.5%), *blaSHV* (25%), *blaOXA* (37.5%), *tetA* (37.5%), *tetB* (25%), *aadA* (37.5%), *sul1* (25%), *MOXM* (12.5%), *DHAM* (25%), and *blaCMY-2* (50%) (Supplementary Figure 1 (SF1)). The genes *blaIMP* and *blaNDM* were not reported in any of the isolates. In this study, 12.5% of the isolates showed co-resistance to colistin and carbapenem.

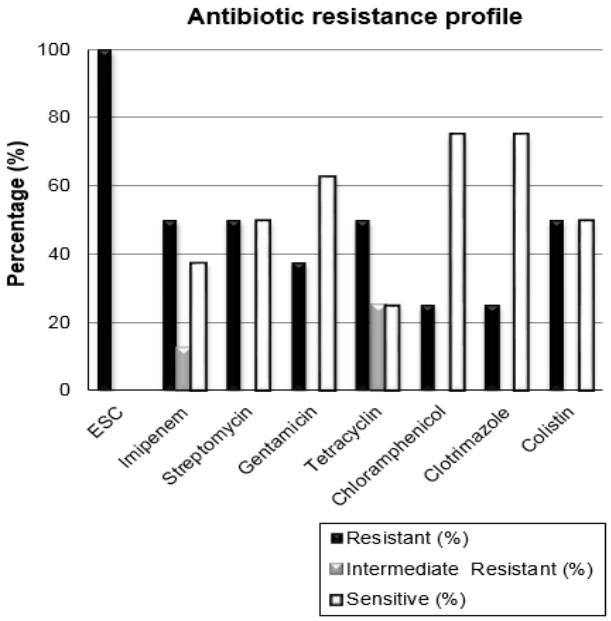

Fig. 1: Antibiotic resistance profile of extended-spectrum beta-lactamase-producing *E. coli* isolates. ESC: Extended spectrum cephalosporin

Table 2: Phenotypic characterization of antimicrobial resistance

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Ampicillin-sulbactam	100	0	0
Amoxicillin plus clavulanic acid	100	0	0
Cefazoline	100	0	0
Cefixime	100	0	0
Cefoxitin	100	0	0
Cefoperazone	100	0	0
Ceftriaxone	100	0	0
Cefotaxime	100	0	0
Ceftazidime	100	0	0
Aztreonam	100	0	0
Imipenem	50	12.5	37.5
Streptomycin	50	0	50
Gentamicin	37.5	0	62.5
Tetracycline	50	25	25
Chloramphenicol	25	0	75
Clotrimazole	25	0	75
Colistin	50	0	50

Table 1: Details of samples collected from various districts of Punjab

Sampling district	Mastitic milk	ESBL detected	% (District wise)	Healthy milk	ESBL detected	% (District wise)
Ludhiana	18	2	11.11	5	0	0
Moga	76	4	5.26	11	0	0
Ferozpur	31	2	6.45	4	0	0
Total	125	8	6.4	20	0	0

Discussion

The present study revealed that the ESBL-producing isolate showed higher resistance to the beta-lactam group of antibiotics, including all the third-generation cephalosporins (P<0.05). Among the AMR genes conferring resistance to the beta-lactam group of antibiotics, *blaCTXM* is most commonly reported in *E. coli*, followed by *blaTEM* and *blaSHV*. The highest resistance against the beta-lactam group of antibiotics signifies their widespread use in livestock. The history collected through questionnaires showed that the beta-lactam group of antibiotics is more prominent among goat farmers. The beta-lactam genes responsible for conferring resistance to various beta-lactam groups of antibiotics originate from primitive penicillin-binding protein (PBP). High resistance to these antibiotics suggests that these genes may have failed to evolve with time to cope with the novel antibiotics and analogues that have come up in the market, thus failing the race of survival of the fittest. None of the isolates possessed the Metallo beta-lactamase genes. A possible reason may be less usage of carbapenems in the livestock sector due to high cost. A significant association between the (class D beta-lactamase) *blaOXA* gene and imipenem resistance suggests that carbapenemase production by the isolates is mainly responsible for resistance. Among the plasmid-mediated *AmpC* beta-lactamase genes, *blaCMY-2* is the most commonly reported, and *MOXM* is the least prevalent among isolates. Besides the beta-lactam group of antibiotics, tetracyclines are also widely used in livestock practice. However, on the contrary, tetracycline resistance is seen only in half of the ESBL-producing *E. coli* isolates. Among the tetracycline resistance genes, *tetA* is more prevalent than *tetB*. As no significant association was noted for the *aadA* gene, isolates with phenotypic resistance to streptomycin and a negative genotype may possess other genes that confer the desired resistance. In isolates, possessing an *aadA* gene with a negative phenotype may signify that the gene did not express itself despite being present. However, such isolates can pass the genes to a related or different organism where the genes can actively confer resistance to a different organism. The most commonly occurring beta-lactam gene detected in *E. coli* is *blaCTXM*. The study results are similar to the findings of various authors (Paterson and Bonomo, 2005; Peymani *et al*., 2017) who found that *blaCTXM* is the most prevalent beta-lactam gene in *E. coli*. The results obtained from this study are similar to the findings of previous studies (Doi *et al*., 2007; Enne *et al*., 2008; Gousia *et al*., 2011; Tong *et al*., 2015). Colistin is the drug of last resort and is advised only when the other treatment options failed. This drug is banned for use in livestock. The emergence of carbapenem and colistin-resistant bacteria puts an additional threat to global drug resistance.

Mastitis is one of the most prevalent and costly diseases in livestock farming, and drug resistance is an emerging global health concern. In a study conducted by Coates *et al*. (2011), it was observed that the majority of antibiotic classes (20 classes) available for *Enterobacteriaceae*, along with their analogues came into existence between 1940-1962, and this was sufficient to sustain the world for 60 years after that. If the present trend of the ongoing emergence of drug-resistant bacteria continues, there may come a point when the world faces an antibiotic crisis.

Acknowledgement

Special thanks of gratitude to Mr. B. K. Satpathy for his assistance with statistical analysis.

Conflict of interest

The authors declare that there is no conflict of interest.

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Supporting Online Material

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