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Molecular Characterization and Antimicrobial Resistance Profiling of Extended Spectrum Beta-lactamase (ESBL) Producing *Escherichia coli* in Bovines from J and K, India

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ABSTRACT

Background: Higher prevalence of ESBL producers is alarming in dairy sector. So limiting antimicrobial use may curtail the selection and persistence of predominant ESBL and conjugative plasmids among strains. The study was aimed to determine the occurrence of extended-spectrum beta-lactamase (ESBL) producing *E. coli* as well as their genetic diversity, antimicrobial resistance and integrons in bovines.

Methods: A total of 180 faecal samples were screened for the presumptive ESBL producing *E. coli* isolates. All ESBL producing isolates were subjected to the screening test and were confirmed as ESBL producers by double disc. Further, these ESBL producers were tested for the presence of *bla* genes and those found positive were tested for multidrug resistance (MDR) by disk diffsion. MDR positive isolates were further tested for the presence of *intI* 1, *intI* 2 and *intI* 3 genes.

Result: A total of 360 presumptive ESBL producing *E. coli* isolates were obtained. Out of which, 154 (42.77%) isolates were found to be resistant and were confirmed as ESBL producers. Of 154 ESBL isolates, 120 (77.92%) isolates carried the gene/s screened for bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes are were declared as multi drug resistant. Out of 120 MDR isolates, 59 tested positive for the integron gene.

Key words: *bla*, Cephalosporin, Integron, Multi drug resistance.

INTRODUCTION

Antimicrobial resistance (AMR) is a growing problem in veterinary medicine because it involves many different species of animals and microorganisms, as well as different animal rearing environments and resistance mechanisms. Some of the most common pathogenic bacteria, such as *E. coli* and *Staphylococcus*, are becoming increasingly resistant to first-line antibiotics (Lewis *et al*., 2007; Pop and D'Agata, 2005). Extended spectrum beta-lactamases are the enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (*e.g.*, ceftazidime, cefotaxime and ceftriaxone) and monobactams (*e.g.*, aztreonam) but do not affect cephamycins (*e.g.*, cefoxitin and cefotetan) or carbapenems (*e.g.*, meropenem or imipenem). The majority are derivatives of the TEM and SHV β-lactamase families, while others, including CTX-M, OXA and KPC β-lactamases, have only recently been discovered. The main mechanism underlying the rise of antibiotic resistance is horizontal gene transfer by mobile genetics elements like plasmids and integrons (Correa *et al*., 2014). Integrons are DNA elements that allow bacteria to share antibiotic resistance genes (Kargar *et al*., 2014). Integrons, also known as gene cassettes, are genetic components that receive and exchange foreign DNA through a site-specific recombination mechanism (Stokes and Hall, 1989). The most well-known gene cassettes discovered within integrons are antibiotic resistance gene cassettes. The increased incidence of MDR bacteria has prompted a frenzy of research on the genetics and methods by which bacteria have evolved antimicrobial drug resistance. There are three types of integrons: *intI*1, *intI*2 and *intI*3, all of which have been linked to antibiotic resistance genes (Mazel *et al*., 2006). Over 130 resistance gene cassettes have been found to be encoded by Class 1 integrons. In class 2 integrons, however, only 6 cassettes have been identified. In the literature and the GenBank database, there is also a lack of variety in class 3

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integrons (Correa *et al*., 2014). Accurate prevalence estimates of ESBL-producing *E. coli* are currently unavailable due to the lack of regional or nationwide surveillance programmes.

MATERIALS AND METHODS

Sample collection

A total of 200 (of which 180 were *E. coli*) faecal samples were collected from healthy cattle and buffalo from different areas of Jammu region between the period from March 2019 to January 2021.

Phenotypic tests for the detection of ESBLs

Isolation of presumptive ESBL producing *Escherichia coli*

The faecal samples were inoculated into nutrient broth and incubated at 37°C until the suspension matching 0.5McFarland standard (1.5×10^8 CFU/mL) and 10 µl of this suspension was spread on ESBL ChromoSelect Agar plates using sterile spreader. Two pink colonies were selected from each plate and streaked on the nutrient agar slant separately, for further screening.

Screening of presumptive ESBL producing *E. coli* for resistance to ceftazidime and cefotaxime by disk diffusion test

The ESBL isolates were subjected to screening for resistance to cefotaxime and ceftazidime by disk diffusion test as recommended by CLSI. A suspension of each isolate matching 0.5 McFarland standard was made in nutrient broth. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, the cefotaxime and ceftazidime antibiotic disks were placed on the surface and the plates were incubated at 37°C for 18-24 hours. Following growth, the diameter of the zone of inhibition around the disks were measured and recorded. Isolates showing resistance to at least one of the antibiotics were considered for further processing.

Confirmation of ESBL producing *E. coli* by cephalosporin/clavulanate combination disks

Isolates of *E. coli* that were resistant to cefotaxime and/or ceftazidime were subjected to phenotypic confirmatory test by using Double Disks Synergy Test as recommended in 2010 by CLSI guidelines which advocates use of ceftazidime (30 µg) (CAZ), ceftazidime + clavulanic acid (30/10 µg) (CAC), cefotaxime (30ìg) (CTX), cefotaxime + clavulanic acid (30/10 µg) (CEC) discs. An increase in the zone diameter by ≥5 mm around the disks containing cephalosporin with clavulanic over the disks containing cephalosporin alone confirmed ESBL production.

Molecular characterization of ESBL producing *E. coli* isolates

Extraction of bacterial DNA

The DNA was isolated by snap and chill method which includes boiling of colonies suspended in distilled water for 10 min to release DNA, cooled on ice for 10 min and centrifuged at 10,000 × g for 1 min.

Detection of ESBL producing *E. coli* isolates

All the isolates found positive for ESBLs production phenotypically, were tested for the presence of bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{OXA} genes by PCR assay (Fang *et al*., 2008). About 10 microlitres of PCR product was electrophoresed in a 1% (w/v) agarose gel for 1 hr at 5 V/cm with a Standard molecular weight marker.

Antimicrobial susceptibility testing

Bacterial isolates found to be positive for ESBL genes by m-PCR, were tested for multidrug resistance by the disk diûusion method in accordance CLSI,2010 guidelines against 20 antibiotics (Table 1). Zone of inhibition were measured and the susceptibility (or resistance) of each isolate was determined.

Molecular detection of class 1, 2 and 3 Integrons

Multi drug resistant ESBL positive isolates were tested for the presence of *intI* 1, *intI* 2 and *intI* 3 genes by m- PCR (Machado *et al*., 2005).

RESULTS AND DISCUSSION

Isolation of presumptive ESBL producing *Escherichia coli*

A total of 360 presumptive ESBL producing *E. coli* isolates (2 from each sample) were obtained from 180 faecal samples. Present study revealed that the total prevelance of ESBL producing *E. coli* in bovines of Jammu region is 42.77% and is reported for the first time in Jammu region. The higher prevalence rate recorded in the current study could be attributed to the indiscriminate use of 3rd generation cephalosporins as a source of growth promoters and disease prevention in bovines, as well as the Plasmid-mediated horizontal transfer of the *bla* gene. This study revealed higher prevelance when compared to other studies, where it is recorded as 29.1% from Andhra Pradesh by Sharif *et al*. (2017) and 35% from Assam by Borah *et al*. (2014). As analyzed from the different reports, the prevalence rate of ESBL in bovine has increased systematically from 35% in 2014 to 42.77% in the present time in India. When the scenario in India is compared with the worldwide scenario, the frequency detected in present study is comparable to 43.6% from china (Zheng *et al*., 2018), 47.7% from Nepal (Subramanya *et al*., 2021), but higher than 4.8% from Malaysia (Kamaruzzaman *et al*., 2020) and 11.2% from Germany (Michael *et al*., 2017), However highest percentage (63.2%) was reported by Olowe *et al*. (2015) from Nigeria.

Screening of presumptive ESBL producing *E. coli* for resistance to ceftazidime and cefotaxime by disk diffusion test

A total of 154 (42.77%) isolates were found to be resistant. Resistance to cefotaxime and ceftazidime was observed in 94 isolates (61.03%) and 60 isolates (38.96%), respectively

and 70 (45.45%) isolates showed resistance to both as depicted in Fig 1. In contrast to findings of Faruk *et al.* (2016) from Turkey, reported diminutive sensitivity of 11.11% and 2.22% of isolates against cefotaxime and ceftazidime, respectively. 70 (45.45%) isolates showed resistance to both the antibiotics. The increased sensitivity of *E. coli* isolates to ceftazidime, cefotaxime and ceftriaxone in this study could be attributed to the fact that third generation cephalosporins are more active against Gram negative organisms (Karchmer, 1995).

Screening for ESBL production by double discs synergy test

All the 154 isolates were confirmed as ESBL producers based on the Double Discs Synergy Test as shown in Fig 2.

Detection of *bla* genes by PCR

Out of 154 ESBL isolates, only 120 (77.92%) isolates carried bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes. Of these only 04 (3.33%) isolates carried bla_{TEM} gene alone, 65 (54.16%) isolates carried bla_{CTX-M} gene alone, 45 (37.50%) isolates carried both bla_{TEM} /bla_{CTX-M} genes and only 06 (5.0%) isolates carried bla_{SHV} /bla_{TEM} /bla_{CTX-M}. bla_{OXA} gene could not be detected in any of these 120 isolates. Fig 3 showed genes amplified from m-PCR assay. In contrast to this study, Borah *et al.* (2014) from Assam and Sharif *et al.* (2017) from Andhra Pradesh reported the bla_{CTX-M}, bla_{SHV} and/or bla_{TEM} type ESBLs in cattle. $bla_{CTX-M-15,}$ bla_{TEM-52} and bla_{SHV-12} have been reported from Germany by Michael *et al.* (2017). According to the findings, bla_{CTX-M} is the most common ESBL type in cattle of Jammu region, with *E. coli* being the most common ESBL producer. The higher rate could be attributed to the widespread use of third-generation cephalosporins, particularly ceftriaxone and cefotaxime, or it could be linked to high encoding gene mobility.

Table 1: Number of susceptible (S), intermediate (I) and resistant (R) strains of ESBL positive isolates.

Antimicrobials	Cattle (94)			Buffalo (26)		
	S	I	R	S	I	R
Amikacin	15	25	54	0	11	15
Ampicillin	0	0	94	0	0	26
Amoxicillin/clavulanic acid	15	0	79	0	0	26
Aztreonam	22	0	72	0	0	26
Ceftriaxone	13	0	81	13	0	13
Cefexime	0	0	94	0	0	26
Cefepime	0	22	72	0	0	26
Cefoperazone	15	0	79	0	0	26
Chloramphenicol	80	4	10	19	0	7
Ciprofioxacin	7	12	75	0	5	20
Doxycycline hydrochloride	29	5	60	9	0	17
Enrofloxacin	5	8	81	0	0	26
Gentamicin	5	69	20	0	22	4
Imipenem	73	15	6	21	5	0
Kanamycin	0	20	74	0	0	26
Nalidixic acid	9	17	68	18	2	6
Neomycin	0	0	94	0	0	26
Streptomycin	0	7	87	0	0	26
Tetracycline	25	5	64	11	0	14
Trimethoprim	22	0	72	19	0	7

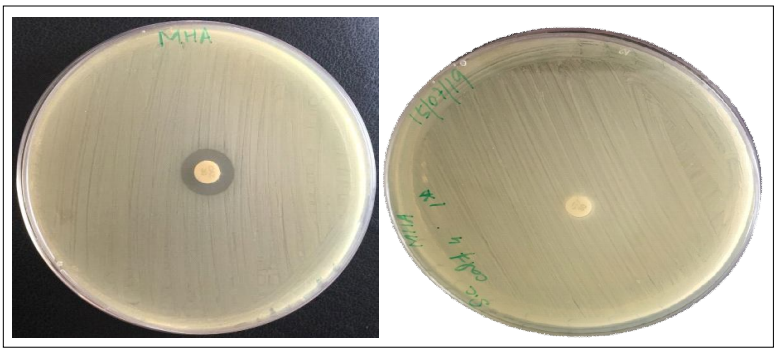

Fig 1: Resistance to ceftazidime (Left) and cefotaxime (Right) by disk diffusion method.

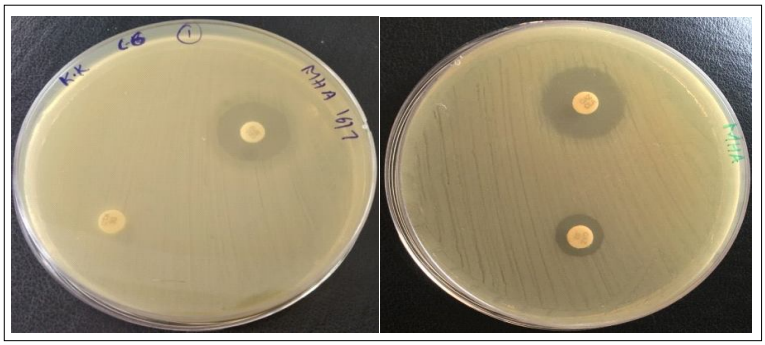

Fig 2: Phenotypic confirmation of ESBLs production in *E. coli* isolates by disk diffusion method using cefotaxime and cefotaxime+clavulanic acid disks(Left) and ceftazidime and ceftazidime+clavulanic acid disks (Right).

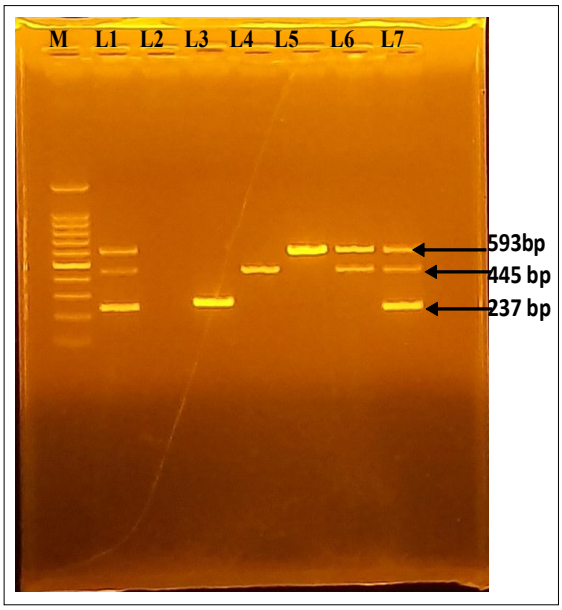

Fig 3: Extended Spectrum beta lactamase genes using multiplex PCR assay:-Lane M: 100 bp DNA Ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3: bla_{SHV} positive, Lane 4: bla_{TEM} positive, Lane 5: bla_{CTX-M} positive, Lane 6: bla_{TEM} and bla_{CTX-M} positive, Lane 7: bla_{SHV}, bla_{TEM} and bla_{CTX-M} positive.

Antimicrobial susceptibility testing

The prevalence of AMR among the ESBL positive strains isolated from cattle and buffalo is shown in Table 1. All 120 *E. coli* isolates showed resistance to at least 20 antibiotics. The isolates that were resistant to more than two classes were identified as multidrug resistant (MDR) isolates. In this study, the prevalence of Ampicillin resistance in ESBL positive isolates was high, which is in agreement with statement from Indonesia by Sudarwanto *et al.* (2016) and from China by Zheng *et al*. (2018). The major finding in the present is the presence of multi drug resistance commensal *E. coli* in bovines to commonly used antibiotics such as amoxicillin/clavulanic acid, aztreonam, ceftriaxone, cefexime, cefepime, enrofloxacin, kanamycin and neomycin, which is comparable to the findings of Borah *et al*. (2014) from Assam, India. The observation of present study was comparable to those of Zheng *et al*. (2018) from China, Ejaz *et al*. (2021) from Brazil and Subramanya *et al*. (2021) from Nepal. It reiterates the finding in other studies that have reported antibiotic resistance among bacteria especially *E. coli* isolated from cattle and other animals is increasing at an alarming rate. However, in our study most of the isolates were senisitive to Imipenem, which is in agreement with Ejaz *et al*. (2021) from Brazil.

Molecular detection of class 1, 2 and 3 integrons

Out of the 120 MDR isolates, 59 were tested for the presence of integron gene. Among these isolates, class 1 integron-encoded *intI* 1 integrase gene was detected in 52 (88.23%) isolates. While 2 (3.38%) isolates tested positive for class 2-encoded *intI* 2 integrase and five isolates harboured both *intI* 1 and *intI* 2. No class 3 integron was detected (Fig 4). The prevalence of *intI* 1 in cattle and buffalo was 95.65% and 100.0%, respectively and the prevalence of *intI* 2 for cattle and buffalo was 10.86% and 15.38%, respectively. The observations are comparable to the 50% in Australia (Barlow *et al*., 2004) but higher than 16.77 % from Korea (Hasan, 2010), 6% from Iran (Kheiri *et al*. 2016). However higher percentage (84.5%) was reported by Ejaz *et al*. (2021) from Brazil. Kheiri *et al*. (2016) from Iran detected class 2- integrase gene in less than 1.2% of isolates. While in our work this percentage was 3.38%, which is in agreement with 4% from Iran by Kheiri *et al*. (2016). ESBL harbour both *intI* 1 and *intI* 2 which is higher than (0.4%) by Kheiri *et al*. (2016) from Iran. Integrons are known to be primary source of transferable resistance genes and are suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (Collis *et al*., 2002). Because integrons have the ability to capture and collect gene cassettes, there is a chance that antibiotic-resistant genes will become common in nature. *E. coli*, which are deadly pathogens if they become antibiotic resistant, can be extremely hazardous to the environment.

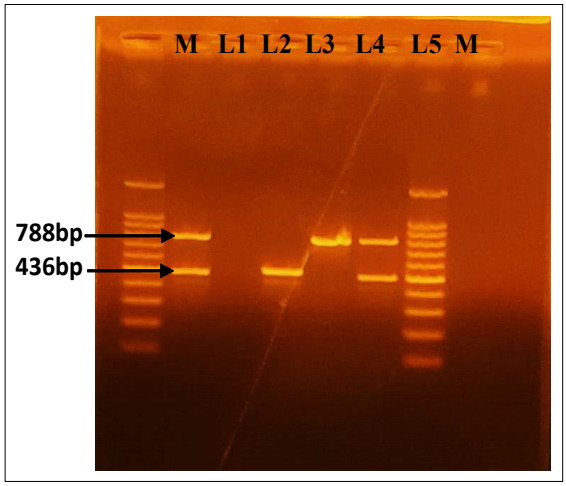

Fig 4: Multiplex PCR (m- PCR) assay for detection of *intI* 1and *intI* 2 genes:- Lane M: 100 bp DNA Ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3: *intI* 1 gene positive, Lane 4: *intI* 2 gene positive, Lane 5: *intI* 1and *intI* 2 genes positive.

CONCLUSION

Antibiotic discovery and development was unquestionably one of the most significant advances in modern medicine. Antimicrobial drug resistance in Gram negative enteric bacteria has emerged as a significant issue in both human and veterinary medicine. The use of antibiotics in farm animals, which are critical in human medicine, has been linked to the emergence of new strains of multi-drug resistant (MDR) bacteria that infect humans. Thus there is an urgent need to focus on the antibiotic selection and to reduce the spread of these increasingly resistant pathogens.

Conflict of interest: None.

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