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Antimicrobial resistance and phenotypic and molecular detection of extended-spectrum β-lactamases among extraintestinal *Escherichia coli* isolated from pneumonic and septicemic sheep and goats in Rajasthan, India

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Abstract: The present study was planned to isolate *Escherichia coli* from pneumonic and septicemic sheep and goats and to determine antimicrobial resistance (AMR) patterns of the isolates and evaluate them for the presence of extended-spectrum β-lactamase (ESBL) using the phenotypic and molecular methods. Pneumonic lung tissues and heart blood samples were collected by performing necropsy on sheep (n = 96) and goats (n = 08). The samples were processed and used for bacterial isolation. *E. coli* isolates (n = 58) including 53 from 34 sheep and 5 from 3 goats were recovered and identified by the cultural and biochemical characteristics and 16S rRNA sequencing. The isolates were tested to ascertain AMR pattern, plasmid features, and ESBL production. The highest rate of resistance (65.5%) was recorded against amoxicillin, enrofloxacin, norfloxacin, and ofloxacin. Eighteen (31%) out of 58 isolates showed the ESBL phenotype by combination disk assay. There was a variability in the number (1–5) and size (2 to >20 kb) of plasmids among isolates. Polymerase chain reaction (PCR) amplification revealed the presence of bla_{TEM} (70.6%) and bla_{SHV} (1.7%) genes. PCR specificity was confirmed by nucleotide sequencing. The present study indicates that the extraintestinal and multidrug-resistant (MDR) *E. coli* strains were associated with pneumonia and/or septicemia in sheep and goats. Therefore, thorough surveillance and monitoring of MDR bacteria are urgently needed to implement the infection control strategies and to restore the efficacy of available antibiotics.

Key words: *Escherichia coli*, extended-spectrum β-lactamase, multidrug resistance, pneumonia, sheep, septicemia

1. Introduction

Pneumonia in sheep and goats is one of the most important diseases of small ruminants. It causes high mortality in lambs leading to significant economic losses for the sheep industry [1]. Pneumonia in sheep and goats develops due to the interaction between environmental factors, microorganisms, and immunity of the host [2]. A number of bacteria like *Mannheimia haemolytica*, *Pasteurella multocida*, *Escherichia coli*, *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp., and *Acinetobacter* spp. have been implicated to cause pneumonia in sheep [3,4]. Annual data reports of Central Sheep and Wool Research Institute (ICAR-CSWRI) farms over two decades indicated high prevalence (15–30%) of pneumonia in sheep populations irrespective of the animal age, and respiratory diseases were the leading cause of mortality in sheep. Enteroinvasive *E. coli* strains invade the intestinal barrier and cause septicemia, particularly in young lambs. They colonize various organs, including the lungs, and cause pathological alterations. The members of *Enterobacteriaceae* are commensals in the intestines of animals and are usually under selective pressure due to the use of antibiotics. Beta-lactams are the most widely used antibiotics against bacterial infections [5]. However, bacterial β-lactamases degrade the β-lactam ring of these antibiotics and result in the development of β-lactam resistance among bacteria. The extended-spectrum β-lactamases (ESBLs) target penicillins and their complexes (amoxicillin-clavulanic acid), monobactams (aztreonam), and up to third-generation cephalosporins [6]. The purpose of the present study was to determine the prevalence of *E. coli* associated with ovine and caprine pneumonia and septicemia and to characterize these isolates based on their antimicrobial resistance (AMR) pattern, ESBL phenotype, plasmid features, and ESBL genes (bla_{TEM} and bla_{SHV}).

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2. Materials and methods

2.1. Geography of the area and disease overview

The study area is located at 26.28°N latitude and 75.38°E longitude in the Tonk district of Rajasthan state, which comes under the semiarid eastern plain. The animals were maintained at the organized sheep and goat farms of ICAR-CSWRI, Avikanagar. At these farms, the animals grazed in the fields during the daytime. In the evening, the animals returned back to the shed where they were provided with dry and green fodder and the formulated concentrates. Pneumonia was the leading cause of mortality at the farms [7,8]. Clinically, the sheep and goats exhibited coughing, sneezing, nasal discharge, and labored breathing. The pneumonic animals showed loss of activity, anorexia, and pneumonic grunts followed by the unexpected death of the animals, particularly the lambs and kids [9]. However, chronic and suppurative pneumonia was also prevalent among adults or aged animals. After the development of clinical pneumonia, the surviving animals became chronically ill with reduced productivity.

2.2. Collection and processing of tissue samples

The lung tissues were aseptically collected in sterile plastic vials by performing necropsy on sheep (n = 96) and goats (n = 8) that died because of pneumonia and septicemia during 2015–2016. Simultaneously, the heart blood samples were also collected directly into sterile syringes. The samples were immediately brought to the laboratory. The tissue samples were processed by making 20% (w/v) tissue homogenate in sterile phosphate-buffered saline and used for isolation of bacteria.

2.3. Bacterial isolation and identification

About 0.5 mL of each tissue homogenate and heart blood was separately inoculated into 10 mL of nutrient broth (HiMedia Pvt. Ltd., Mumbai, India). The broth was incubated at 37 °C for 24 h followed by streaking the broth culture on nutrient agar plates. Developed bacterial colonies were streaked on MacConkey agar (HiMedia Pvt. Ltd.) plates. Lactose-fermenting colonies from MacConkey agar plates were streaked on eosin methylene blue agar (HiMedia Pvt. Ltd.) for the development of metallic sheen specific to *E. coli* isolates. Bacterial isolates were further identified on the basis of biochemical tests, namely indole, methyl red, Voges-Proskauer, and citrate (IMViC), as described by Sleigh and Duguid [10].

The isolates presumptively identified as *E. coli* were streaked on nutrient agar (HiMedia Pvt. Ltd.) and incubated aerobically at 37 °C for 24 h to get fresh colonies for isolation of the genomic DNA. About 4–5 medium sized colonies of each of the bacterial isolates were transferred into plastic tubes of 1.5 mL. The bacterial colonies were washed twice in nuclease-free water by centrifugation at 10,000 × *g* for 3 min in a microcentrifuge. The bacterial genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions and used for PCR amplification of 16S rRNA.

2.4. Antimicrobial susceptibility testing (AST)

The AST of the bacterial isolates was carried out by disk diffusion method using amoxicillin-clavulanic acid (20/10 µg), ceftazidime (30 µg), cefixime (5 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), norfloxacin (10 µg), and tetracycline (30 µg) disks (HiMedia Pvt. Ltd.) as per the CLSI guidelines [11]. To test the susceptibility of *E. coli* isolates to amoxicillin (30 µg), enrofloxacin (5 µg), and chlortetracycline (30 µg) without specific CLSI criteria, a relevant CLSI criterion described for the same class of antimicrobials was used. The procedure of AST was optimized using *E. coli* ATCC 25922 as a reference strain.

2.5. Phenotypic detection of ESBL

To detect the ESBL phenotype of bacterial isolates, a combination disk test was carried out as per the CLSI guidelines [11]. The test was performed on Mueller-Hinton agar (HiMedia Pvt. Ltd.) plates using ceftazidime (30 µg) and cefotaxime (30 µg) disks alone and in combination with clavulanic acid (10 µg). An increase in zone diameter of ≥5 mm for ceftazidime or cefotaxime tested in combination with clavulanic acid versus their zone when tested alone was considered as confirmatory of the ESBL phenotype of the bacterial isolates.

2.6. Isolation of plasmid DNA

The bacterial isolates were further cultured on MacConkey agar to get the individual colony. A single colony of each bacterial isolate was inoculated into 10 mL of Luria-Bertani (LB) broth (HiMedia Pvt. Ltd.) medium kept in sterile plastic tubes of 50 mL and incubated at 37 °C for 12–15 h with agitation in an incubator shaker. The broth suspension (2 mL) was centrifuged at 10,000 × *g* for 3 min in a microcentrifuge to pellet the bacterial cells. The bacterial cells were subjected to plasmid DNA isolation using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Vilnius, Lithuania). The plasmid DNA of each isolate was tested for the amplification of the ESBL genes using PCR.

2.7. Polymerase chain reaction (PCR)

PCR reactions were performed in a peqSTAR 96 universal gradient thermocycler (Peqlab Biotechnology, Erlangen, Germany) using specific primers (Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, India) to amplify 16S rRNA [12] and bla_{TEM} and bla_{SHV} genes [13] (Table). The PCR reaction mixtures and the amplification conditions were optimized for all the genes. For PCR amplification, about 50 ng (2 µL) of the template DNA (genomic DNA for 16S rRNA, plasmid DNA for *bla* genes) was added

Table. Primer pairs used for amplification of different β-lactamase genes.

Genes	Primers	Amplicon size (bp)	Reference
16S rRNA	27F 5`AGAGTTTGATCMTGGCTCAG3`	~1466	[12]
	1492R 5`CGGTTACCTTGTTACGACTT3`		
bla$_{TEM}$	F- 5` CTTCCTGTTTTTGCTCACCCA 3`	717	[13]
	R- 5` TACGATACGGGAGGGCTTAC 3`		
bla$_{SHV}$	F- 5`TCAGCGAAAAACACCTTG 3`	472	
	R- 5`TCCCGCAGATAAATCACC 3`		

to make 20 µL of reaction mixture containing 200 µM of dNTPs, 0.2 µM of each primer, 1.875 mM of $MgCl_2$, and 1 U of Taq DNA polymerase (Sigma-Aldrich Chemicals Pvt. Ltd.) in 1X PCR buffer. The *bla*$_{TEM}$-positive *E. coli* strain (maintained at the laboratory) and the SHV-producing reference strain *Klebsiella pneumoniae* ATCC 700603 were used as the positive control for the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, respectively. *E. coli* ATCC 25922 was used as a negative control for the PCR reactions. After dispensing the master mixture and template DNA, the PCR tubes were spun in a microcentrifuge to mix the reaction components properly. The PCR conditions included an initial denaturation at 94 °C for 3 min followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 50 °C (52 °C for 16S rRNA) for 30 s, extension at 72 °C for 2 min, and the final extension at 72 °C for 5 min. The amplified products (5 µL) were stained with ethidium bromide (0.5 µg/ mL) in agarose gel (1.5%, w/v) and separated in a horizontal submarine electrophoresis unit using 1X TAE as the running buffer, and examined under a gel documentation system (UVP, Upland, CA, USA).

3. Results

By necropsy examination, the petechial and ecchymotic hemorrhagic lesions along with congestion and the varying degree of consolidation were recorded in the lung lobes (Figure 1). Apical, cardiac, and anteroventral parts of the diaphragmatic lobes were found to be commonly affected. The straw-yellow colored mucoid and frothy exudate was seen in the bronchi and bronchioles. Chronic and complicated cases showed suppuration in the lungs. In some carcasses, the lungs were seen to have adhered to the diaphragm and thoracic wall. Hemorrhagic changes were also recorded in the trachea. The tracheal lumen was found to be filled with mucus and froth. Petechial hemorrhages were evident on the epicardium (Figure 1) and spleen.

A total of 58 *E. coli* isolates, including 53 from sheep (n=34) and 5 from goats (n=3), were obtained by bacterial isolation. Besides the *E. coli* isolates, other gram-negative and gram-positive bacteria were also recovered, which were not included for further study. The *E. coli* isolates were presumptively identified by the cultural and biochemical characteristics. They produced medium sized, round, smooth, and white to light grayish colonies on sheep blood agar on which a few isolates also caused partial hemolysis of sheep RBCs.

Resistance was recorded against the multiple antimicrobials of different classes such as penicillins (amoxicillin and amoxicillin-clavulanic acid), cephalosporins (ceftazidime and cefixime), carbapenems (meropenem and imipenem), aminoglycosides (amikacin), fluoroquinolones (ciprofloxacin, enrofloxacin, norfloxacin, and ofloxacin), and tetracyclines (tetracycline and chlortetracycline). The resistance to penicillin (amoxicillin) and fluoroquinolones (enrofloxacin, norfloxacin, and ofloxacin) was recorded at the highest rate (65.5%). Bacterial isolates, which showed resistance to the antimicrobials of 3 or more classes, were categorized as multidrug-resistant (MDR) isolates. However, all MDR isolates showed susceptibility to gentamicin. The antimicrobial resistance profile of the isolates is summarized in Figure 2.

Among the 58 isolates tested by a combination disk assay, 18 (31%) isolates exhibited a zone diameter of ≥5 mm higher when tested with ceftazidime-clavulanic acid and cefotaxime-clavulanic acid compared to ceftazidime or cefotaxime alone, indicating an ESBL phenotype.

There was variability in the plasmid features. The number and size of the plasmids varied among the isolates. Most of the isolates harbored plasmids that ranged from 2 to 20 kb (Figure 3). Larger plasmids of more than 20 kb were also recorded. Twenty isolates did not show any visible plasmids on separation through gel electrophoresis.

The PCR reactions showed amplification of the *bla*$_{TEM}$ gene with an expected amplicon size of 717 bp among 41 (70.6%) isolates including 37 (69.8%) of sheep and 4 (80%) of goats. Eight of 18 isolates with the ESBL phenotype were found to carry the blaTEM gene. The *bla*$_{SHV}$ gene (472 bp) was detected in one (1.7%) isolate (Figure 3). The nucleotide sequencing of the PCR products confirmed the

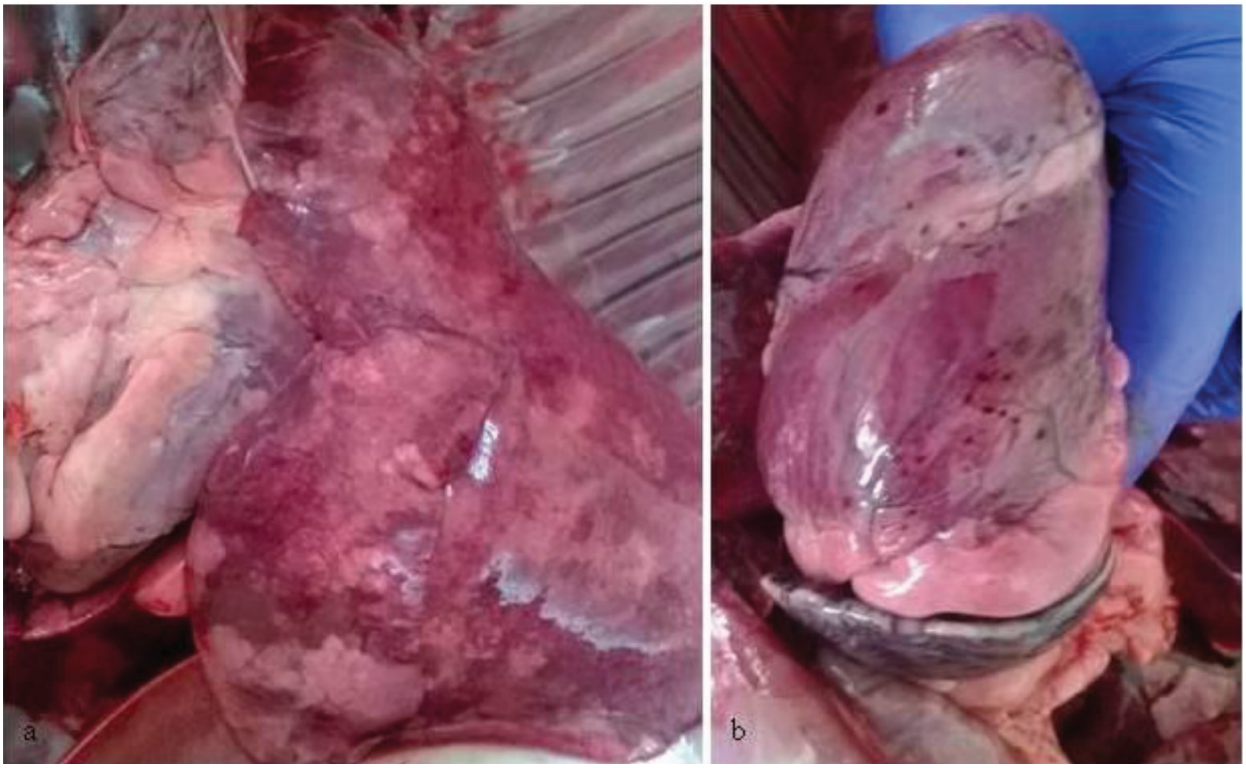

Figure 1. Congestion and consolidation of pneumonic lung (a) and petechial hemorrhagic lesions on epicardium (b).

specificity of the bla_{TEM} and bla_{SHV} genes. Gene sequences have been submitted to the NCBI GenBank database with MK759881.1 (16S rRNA), MK371547.1 (bla_{TEM}), and MK371548.1 (bla_{SHV}) accession numbers.

4. Discussion

Due to the involvement and interplay of multiple factors in respiratory diseases, it is difficult to ascertain the differential pathology of pneumonia [14]. Gross pathological changes in the lungs, in the present study, were similar to the previous reports on ovine bronchopneumonia [2,15]. A high prevalence (~25%) of *E. coli* associated with pneumonia in sheep has been reported earlier [2,16]. Enteroinvasive *E. coli* can invade the intestine and colonize other body organs through blood circulation due to a breach in the immunity of the host [16,17]. The presence of petechial hemorrhages on different organs such as the heart and spleen indicated septicemia. Holmoy et al. [18] recorded that *E. coli* existed among 65% of the septicemic lambs. Ruminants, particularly sheep and cattle, are recognized as reservoirs of *E. coli* (STEC) strains [19].

E. coli is genetically heterogeneous in nature and inhabits the normal microflora of the intestinal tract of animals and humans [20]. However, certain strains of this bacterial species have acquired some genes that enable them to act as systemic pathogens [21]. Further, different *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors, which affect a wide range of cellular processes [21]. Isolation of *E. coli* from pneumonic lungs of sheep might be a sign of secondary bacterial complication as a consequence of primary viral infection. Some viruses play an important role in lamb pneumonia with regard to the onset of infection [22,23]. The correlation between viruses and bacteria with respect to ovine and caprine pneumonia in the present study was not determined.

Recently, *E. coli* isolates which are resistant to multiple antibiotics have been reported frequently [24,25]. A combination disk assay revealed the ESBL phenotype of the bacterial isolates. Several studies applied this assay for phenotypic identification of the ESBL production by *Enterobacteriaceae* [24,25]. The present study describes the occurrence of β-lactamase-producing extraintestinal *E. coli* associated with pneumonia and/or septicemia in small ruminants. Apart from β-lactam resistance, the isolates were also found resistant to non-β-lactam antibiotics. Resistance to multiple antimicrobials among *E. coli* isolates indicates the emergence of multidrug resistance. Phenotypic identification of MDR organisms is important for epidemiological purposes as well as for infection control measures [26]. MDR threats limit the therapeutic use of antibiotics. Therefore, it is important to check regularly the

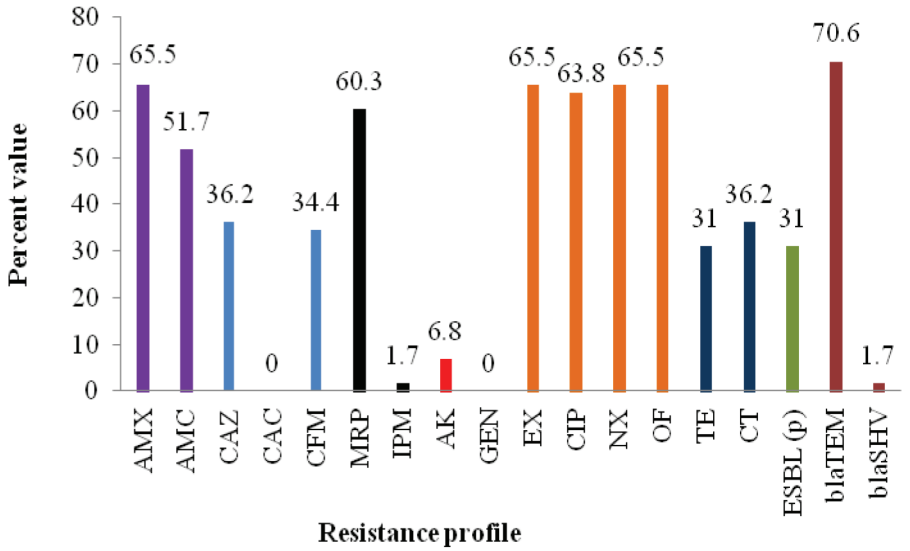

Figure 2. Antimicrobial resistance profile of bacterial isolates showing resistance rates to different antimicrobials and the prevalence of the ESBLs. AMX: Amoxicillin, AMC: Amoxicillin-clavulanic acid, CAZ: Ceftazidime, CAC: Ceftazidime-clavulanic acid, CFM: Cefixime, MRP: Meropenem, IPM: Imipenem, AK: Amikacin, GEN: Gentamicin, EX: Enrofloxacin, CIP: Ciprofloxacin, NX: Norfloxacin, OF: Ofloxacin, TE: Tetracycline, CT: Chlortetracycline, ESBL (p): Prevalence of ESBL phenotype, blaTEM: Prevalence of bla_{TEM} gene, blaSHV: Prevalence of bla_{SHV} gene.

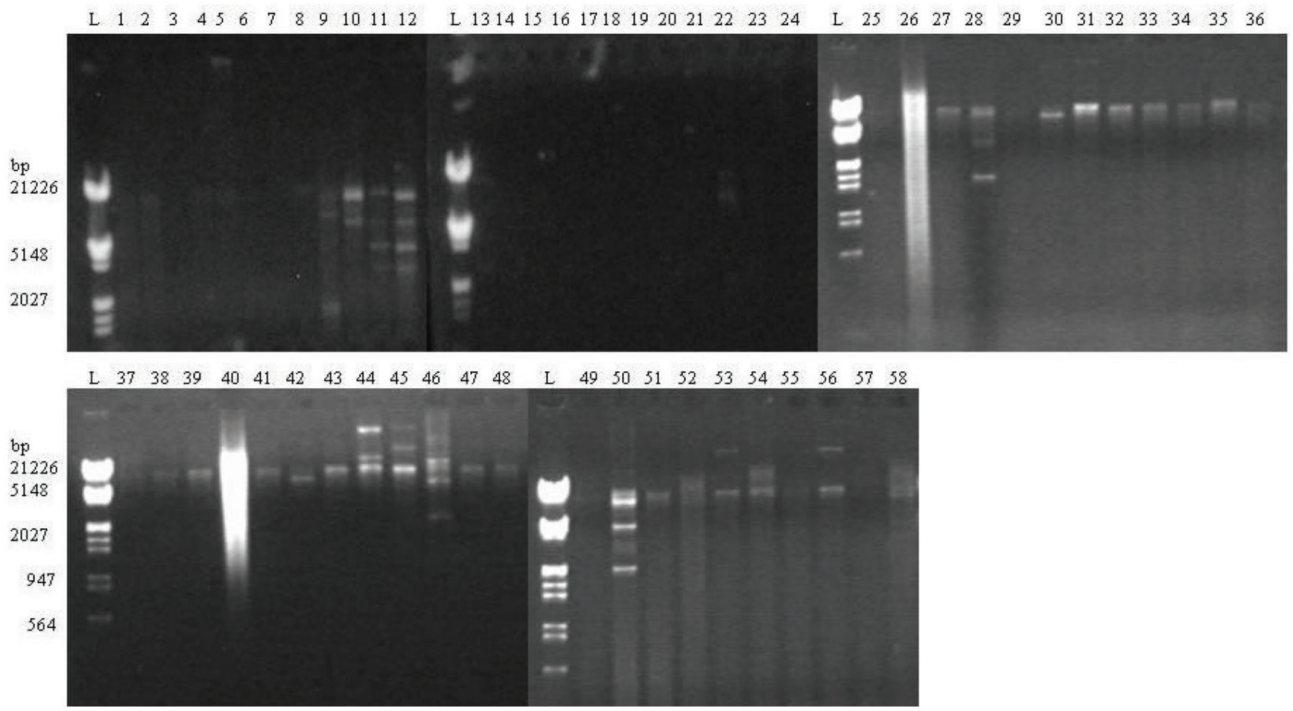

Figure 3. Bacterial isolates showing variability in number (1–5) and size (from ~2 kb to >20 kb) of the plasmids. Lane L: Lambda DNA/ EcoRI + Hind III marker.

emergence of AMR among gram-negative bacteria with the aim to select suitable and effective antimicrobials for therapeutic applications and to control the further spread of resistant pathogens.

The genetic elements of AMR in *Enterobacteriaceae* bacteria are usually located in the plasmids. Due to the selection pressure of antibiotics, the bacteria by means of plasmids may acquire genetic properties to survive in the

presence of antibiotics [5]. Being extrachromosomal with dynamic functions, the bacterial plasmids may exist with variable features. A single bacterium can acquire multiple plasmids from different donors in different environmental conditions. Plasmid features with variable number (1–5) and size (2 to >20 kb) have been reported earlier [27], and thus corroborate the findings of the present study. The β-lactamases such as the ESBLs and AmpC β-lactamases, and inhibitor-resistant derivatives of TEM and SHV, get transferred through the large plasmids [28]. A high prevalence of the blaTEM gene established the genetic basis of β-lactam resistance among the isolates. Further, a higher carriage of the bla_{TEM} gene compared to the ESBL phenotype might be due to the low expression level and/or nonexpression of this gene in phenotypic characters. Moreover, all bla_{TEM} subtypes are not ESBL. However, a low prevalence of bla_{SHV} (1.7%) compared to the higher percentage of ESBL phenotype (31%) among bacterial isolates indicates that these organisms might have also harbored the other ESBL genes that were not focused on in the present study.

Isolation of the ESBL-producing MDR bacteria associated with disease conditions in animals emphasized the zoonotic implications of these resistant organisms [24,25]. At the interface of humans, animals, and the environment, the epidemiology of antimicrobial-resistant bacteria is complex and multifactorial with respect to their transmission and spread [29]. These pathogens might be a new source of infections for human and animal populations by means of their interaction in the environment.

It is concluded that *E. coli* was identified as a major bacterial organism associated with pneumonia leading to high morbidity and mortality in sheep and goats. The findings of the present study suggest that besides *Mannheimia* and *Pasteurella*, which are commonly responsible for the development of bronchopneumonia in small ruminants, *E. coli* could be an important opportunistic pathogen to develop or complicate pneumonia in these animals. As per our knowledge, this might be the first study reporting an occurrence of MDR extraintestinal *E. coli* isolated from lungs and heart blood of food animals such as sheep and goats from India. Moreover, the recovery of such MDR bacteria has zoonotic implications that reflect the urgent need for thorough surveillance and monitoring of these resistant pathogens to restrict their further spread and to restore the efficacy of available antibiotics.

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