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Detection and characterization of extended-spectrum beta-lactamase genes (*bla*$_{TEM}$ and *bla*$_{SHV}$) among beta-lactam-resistant fecal coliforms of dairy cattle from Chhattisgarh, India

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Abstract: The aim of this study was to isolate the antimicrobial-resistant enterobacteria from cattle feces and to identify the extended-spectrum beta-lactamase (ESBL) and carbapenemase gene among them. A total of 70 cattle fecal swab samples were collected from Durg, Chhattisgarh, India, and cultured in nutrient broth. Bacterial isolation recovered 120 gram-negative bacteria. Antimicrobial susceptibility testing showed 90 (75%) coliform isolates (66 *Escherichia coli*, 20 *Klebsiella* sp., and two each of *Enterobacter* sp. and *Citrobacter* sp.) resistant to amoxicillin, amoxicillin-clavulanic acid, cefixime, ceftazidime, imipenem, or meropenem. Bacterial isolates showed the highest resistance rate for ceftazidime (61.6%), followed by cefixime and amoxicillin-clavulanic acid (60%). The ESBL phenotype, assessed by combination disk method, was shown by 36 (40%) of the resistant isolates. PCR amplification revealed 20% and 31.6% positivity of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, respectively. *bla*$_{TEM}$ and *bla*$_{SHV}$ coexpression was recorded among eight (12.1%) *E. coli* and two (10%) *Klebsiella* sp. isolates. Restriction endonuclease analysis indicated clonal *bla*$_{TEM}$ and clonal and nonclonal *bla*$_{SHV}$ genes. Nucleotide sequencing confirmed the *bla*$_{TEM}$ and *bla*$_{SHV}$ genotypes. None of the isolates showed amplification of the carbapenemase gene. Phenotypic and genetic detection and characterization of ESBLs among Enterobacteriaceae bacteria illustrate the importance of planning and execution of effective control programs for emerging antimicrobial-resistant pathogens.

Key words: Beta-lactam resistance, cattle, coliform, Enterobacteriaceae, extended-spectrum beta-lactamase

1. Introduction

One of the most important mechanisms for development of antimicrobial resistance of bacteria is mediated by the production of bacterial beta-lactamases. Extended-spectrum beta-lactamases (ESBLs) are capable of targeting a broader spectrum of antibiotics, including penicillins and their complexes, monobactams, and up to third-generation cephalosporins (1). Members of Enterobacteriaceae are commensals in the intestines of animals and are usually under selective pressure by use of antibiotics for treatment and prevention of infectious diseases (2). Furthermore, *Escherichia coli* and *Klebsiella pneumoniae* expressing ESBLs in particular are important microbial pathogens that have been reported more frequently in association with the acquisition and transmission of drug-resistance elements (3,4). There are limited reports on beta-lactam-resistant enterobacteria related to livestock animals from India. Therefore, the present study was planned to isolate the bovine fecal enterobacteria and to characterize them on the basis of beta-lactam resistance. Isolation of antimicrobial-resistant bacteria from animal sources indicates their possible spread into the environment and/or human populations. ESBL genes can be transferred from nondisease-causing bacteria to those that cause disease. Surveillance of these genes could be a valuable tool to screen the resistance trends at a population level (5). The emergence of antimicrobial resistance is usually assessed either by population analysis or susceptibility testing of bacteria (6). Advance drug resistance surveillance and knowledge of the molecular characteristics of ESBL-producing isolates are necessary to guide the appropriate and judicious use of antibiotics (7).

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2. Materials and methods

2.1. Collection and transportation of samples

A total of 70 fecal swab samples were collected randomly from farmed dairy cows reared in semiorganized herds in the Durg district of the Chhattisgarh state of India during the monsoon season of 2014. All the fecal samples were collected in screw-capped sterile specimen containers and immediately transported to the laboratory for isolation of bacteria.

2.2. Bacterial isolation and identification

Each of the fecal swab samples was separately inoculated into 10 mL of nutrient broth and incubated at 37 °C for 24 h. The broth cultures were streaked onto MacConkey agar (HiMedia Pvt. Ltd., Mumbai, India) and further incubated at 37 °C for 18–24 h to isolate the enterobacteria. Bacterial colonies were screened based on morphological features. Each of the distinct colonies was considered as one isolate and maintained in a pure culture slant. Bacterial identification was done by cultural and biochemical tests following indole, methyl red, Voges–Proskauer, and citrate utilization tests and urease test and H_2S production on triple sugar iron agar (8).

2.3. Antimicrobial susceptibility testing (AST)

AST of bacterial isolates was performed following the disk diffusion method using amoxicillin (30 µg), amoxicillin-clavulanic acid (30 µg), ceftazidime (30 µg), cefixime (5 µg), imipenem (10 µg), and meropenem (10 µg) disks (HiMedia Pvt. Ltd.) (9). The procedure was optimized against *Escherichia coli* ATCC 25922.

2.4. Phenotypic tests for ESBLs and carbapenemases

A combination disk method was used for phenotypic detection of ESBL production by bacteria as per CLSI guidelines (9). The test was performed on Mueller–Hinton agar (HiMedia Pvt. Ltd.) petri plates using disks of extended-spectrum cephalosporins like ceftazidime (30 µg) alone and in combination with clavulanic acid (10 µg). A ≥5-mm increase in zone diameter for ceftazidime tested in combination with clavulanic acid versus its zone when tested alone was considered as confirmatory for ESBL production.

The modified Hodge test was performed for phenotypic assessment of carbapenemase production by carbapenem-resistant enterobacteria (9). *Klebsiella pneumoniae* BAA 1705 (KPC-positive) and *Klebsiella pneumoniae* BAA 1706 (KPC-negative) were used as quality control organisms. The *Escherichia coli* ATCC 25922 strain was used as an indicator organism with a meropenem disk (10 µg) to perform this test.

2.5. Isolation of plasmid DNA

The resistant bacterial isolates were streaked onto MacConkey agar to get a pure colony. A single colony of each isolate was inoculated into 10 mL of Luria–Bertani (LB) broth medium (HiMedia Pvt. Ltd.) kept in 50-mL tubes and incubated at 37 °C for 12–15 h in an incubator shaker. The pellet was prepared by centrifuging the bacterial suspension at 10,000 × *g* for 3 min. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA) and used for detection of antimicrobial resistance genes.

2.6. Polymerase chain reaction (PCR)

PCR amplification was performed in a thermal cycler (Eppendorf, Hamburg, Germany) using specific primers (Sigma Aldrich, St. Louis, MO, USA) (Table) to detect the bla_{TEM} (10), bla_{SHV} (11), bla_{KPC}, and bla_{NDM} (12) genes. The PCR reaction mixtures and amplification conditions were optimized for all genes. The bla_{TEM}-positive *Escherichia coli* (maintained at the laboratory), *Klebsiella pneumoniae* ATCC 700603, *Klebsiella pneumoniae* ATCC BAA-1705, and bla_{NDM}-positive *Shigella* sp. isolate (accession number KR872630.1) were used as positive controls for the bla_{TEM}, bla_{SHV}, bla_{KPC}, and bla_{NDM} genes, respectively. *Escherichia coli* ATCC 25922 was used as a negative control for all the target genes. PCR amplification was carried out using 25-µL reactions containing 200 µM of dNTPs, 0.2 µM of each primer, 1.25 U of Taq DNA polymerase (Sigma Aldrich),

Table. Primer pairs used for amplification of beta-lactamase genes.

Genes	Primers	Amplicon size	Reference
bla_{TEM}	F- 5'AAAATTCTTGAAGACG 3'	1073 bp	10
	R- 5' TTACCAATGCTTAATCA 3'		
bla_{SHV}	F- 5'TCAGCGAAAAACACCTTG 3'	472 bp	11
	R- 5'TCCCGCAGATAAATCACC 3'		
bla_{KPC}	F-5'CATTCAAGGGCTTTCTTGCTGC 3'	538 bp	12
	R- 5'ACGACGGCATAGTCATTTGC 3'		
bla_{NDM}	F- 5'GGTTTGGCGATCTGGTTTTC 3`	621 bp	
	R- 5'CGGAATGGCTCATCACGATC 3`		

and 1.875 mM of $MgCl_2$ in 1X PCR buffer. About 50 ng of DNA was used as a template. The components of the PCR reaction mixture except $MgCl_2$ (1.25 mM) for bla_{KPC} and bla_{NDM} genes were similar to those for the bla_{TEM} and bla_{SHV} genes. The cycling conditions comprised initial denaturation at 94 °C for 3 min followed by 35 cycles each of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min, for the amplification of the bla_{TEM} and bla_{SHV} genes. For the amplification of the bla_{KPC} and bla_{NDM} genes the cycling conditions comprised initial denaturation at 94 °C for 5 min followed by 39 cycles each of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min with final extension at 72 °C for 10 min. The amplified PCR products (5 µL) were separated using 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) by running them in a horizontal submarine electrophoresis unit using 1X TAE as the running buffer and examined under the gel documentation system (Thermo Fisher Scientific).

2.7. Restriction endonuclease (RE) analyses

PCR-amplified bla_{TEM} and bla_{SHV} gene products were digested with *PstI* and *AvaII* endonucleases (Thermo Fisher Scientific) as per the previously reported method (10) with slight modifications. For RE analyses, the PCR products (5 µL) were mixed with 0.5 µL of RE and 2 µL of 10X buffer. The final volume of 20 µL was made up with nuclease-free water. The reaction mixture was spun in a microcentrifuge and incubated at 37 °C for 18 h in a dry bath. The restriction digested products (10 µL) were separated using 2% agarose (Sigma Aldrich) gel stained with ethidium bromide (0.5 µg/mL).

2.8. Gene cloning and sequencing

Plasmid DNA samples representing the bla_{TEM} and bla_{SHV} genes were amplified by *Pfu* DNA polymerase (Thermo Fisher Scientific). The gene products in excised gels were purified by the GeneJET PCR Purification Kit (Thermo Fisher Scientific), eluted in 25 µL of elution buffer, and ligated into a pJET 1.2/blunt cloning vector (50 ng) provided with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). The ligation mixture was incubated at room temperature (22 °C) for 30 min and used directly to transform the *E. coli* ($DH_5\alpha$). Rapid preparation of chemically competent cells was done using the TransformAid Bacterial Transformation Kit (Thermo Fisher Scientific). The competent cell suspension was plated immediately on prewarmed LB agar (HiMedia Pvt. Ltd.) plates containing 50 µg/mL ampicillin. Plates were incubated overnight at 37 °C in an upside-down position for development of transformant colonies. Colony PCR was performed to screen the positive transformants. Gene-specific colonies were selected and grown further overnight in LB broth containing 50 µg/mL ampicillin. Plasmid DNA was further isolated and used for bidirectional nucleotide sequencing with customized Sanger sequencing (GeNei Laboratories Pvt. Ltd., Bangalore, India).

3. Results

A total of 120 isolates were recovered from the 70 fecal swab samples. Isolates were preliminary identified based on cultural characteristics. All the pure isolate slants were kept at 4 °C for further study. Out of 120 bacterial isolates tested by AST, 90 (75%) isolates were identified as resistant (to amoxicillin, amoxicillin-clavulanic acid, cefixime, ceftazidime, imipenem, and/or meropenem). The isolates showed the highest rate of resistance to ceftazidime (61.6%), followed by cefixime and amoxicillin-clavulanic acid (60%) (Figure 1). The least resistance was recorded against imipenem (6.6%). Identification revealed 66 (55%) *E. coli*, 20 (16.6%) *Klebsiella* spp., and two each of *Enterobacter* sp. and *Citrobacter* sp. isolates based on cultural and biochemical characteristics. Out of the 90 resistant isolates, 22 isolates showed beta-lactamase-positive phenotypic test results with amoxicillin and amoxicillin-clavulanic acid. Twenty-eight isolates showed ESBL-positive phenotypic test results with ceftazidime and ceftazidime-clavulanic acid (Figure 2), while eight isolates developed positive combination disk test results with both of the combinations (amoxicillin and amoxicillin-clavulanic acid, and ceftazidime and ceftazidime-clavulanic acid). Thus, a total of 36 (28 + 8) isolates expressed the ESBL phenotype.

Bacterial isolates showed variability in number (1 to 5) and size (1 kb to >21 kb) of plasmids. The size of most of the plasmids ranged from 2 kb to 20 kb. However, plasmids of more than 21 kb were also recorded (Figure 3). Furthermore, some bacterial isolates were found to harbor plasmids of similar profiles. PCR amplification showed specific detection of bla_{TEM} (1073 bp) (Figure 4) and bla_{SHV} (472 bp) genes (Figure 5). Overall positivity of the bla_{SHV} gene was higher (31.6%) than that of the bla_{TEM} gene (20%). The bla_{TEM} gene was harbored by 20 (30.3%) *E. coli* and 4 (20%) *Klebsiella* sp., whereas the bla_{SHV} gene was shown by 24 (36.6%) *E. coli* and 14 (70%) *Klebsiella* sp. isolates. Coexpression of the bla_{TEM} and bla_{SHV} genes was recorded among eight (12.1%) *E. coli* and two (10%) *Klebsiella* sp. isolates. None of the isolates showed detection of the carbapenemase gene (bla_{KPC} or bla_{NDM}).

RE analysis with *PstI* digestion of the bla_{TEM} product (1073 bp) showed uniform fragments of 758 bp and 315 bp (Figure 6A). The RE pattern of the bla_{TEM} gene by double site cutter *AvaII* showed a uniform pattern of fragments of 636 bp, 222 bp, and 215 bp (Figure 6B). *PstI* digestion of the bla_{SHV} gene (472 bp) recorded a common pattern (304 bp and 168 bp) (Figure 7A), except for two PCR products that revealed intact bands. Again, the RE analysis of the

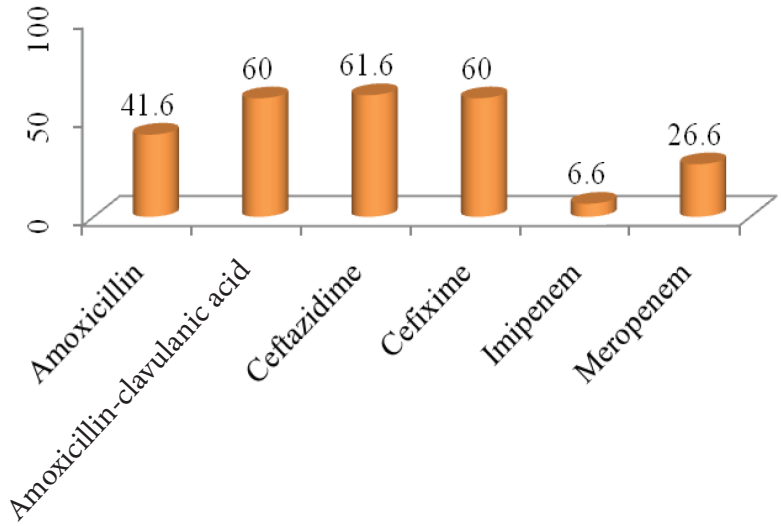

Figure 1. Antimicrobial resistance pattern of bacterial isolates.

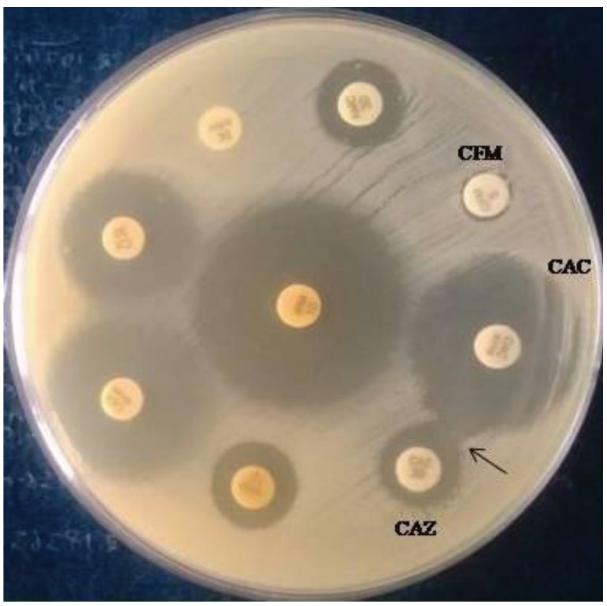

Figure 2. The phenotypic expression of ESBL using combination disk method.

bla_{SHV} gene by *AvaII* reflected the uniform band pattern of DNA fragments of 330 bp and 142 bp (Figure 7B).

Gene sequencing confirmed the bla_{TEM-1} subtype of bla_{TEM} (accession number MF346734) and the bla_{SHV} (accession number MF346732) ESBL genes. Single nucleotide substitutions of T in place of C (T/C) at position 18, A/G at position 138, T/G at position 396, T/C at position 474, and G/A at 717 position were recorded in bla_{TEM}-harboring isolates with respect to the *E. coli* beta-lactamase variant TEM-1D (bla_{TEM-1D}) gene (accession number AF188200.1). In relation to the reference strain (accession number AF188200.1), nucleotide substitutions in the bla_{TEM} gene sequence did not alter the amino acid sequence of the expected translated product (287 amino acids).

4. Discussion

The Enterobacteriaceae are inhabitants of the alimentary tracts of animals and humans and are shed along with the feces into the environment, where they may act as potential threats to public health. Beta-lactam resistance in the present study was recorded among the isolates of the coliform group (*Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter*) of Enterobacteriaceae. Reduced bacterial susceptibility to advanced antimicrobials, namely the carbapenems and the third-generation cephalosporins, may further enhance the uncontrolled spread of these resistant pathogens at the animal–environment–human interface. One of the driving forces behind increased beta-lactam resistance among the Enterobacteriaceae is the use of third- and fourth-generation cephalosporins (13,14). Furthermore, the conjugative nature of enterobacteria facilitates the transfer of beta-lactamase genes from resistant donors to susceptible bacteria.

The present study corroborated the previous reports on ESBL-producing Enterobacteriaceae from bovines in France (15), Switzerland (16,17), the Netherlands (18), Germany (19), and Nigeria (20). There have only been a few reports describing the prevalence and characteristics of ESBL-producing Enterobacteriaceae in healthy farm animals worldwide. To date, no prevalence data are available on ESBL-producing Enterobacteriaceae from cattle in India. However, a negative correlation between

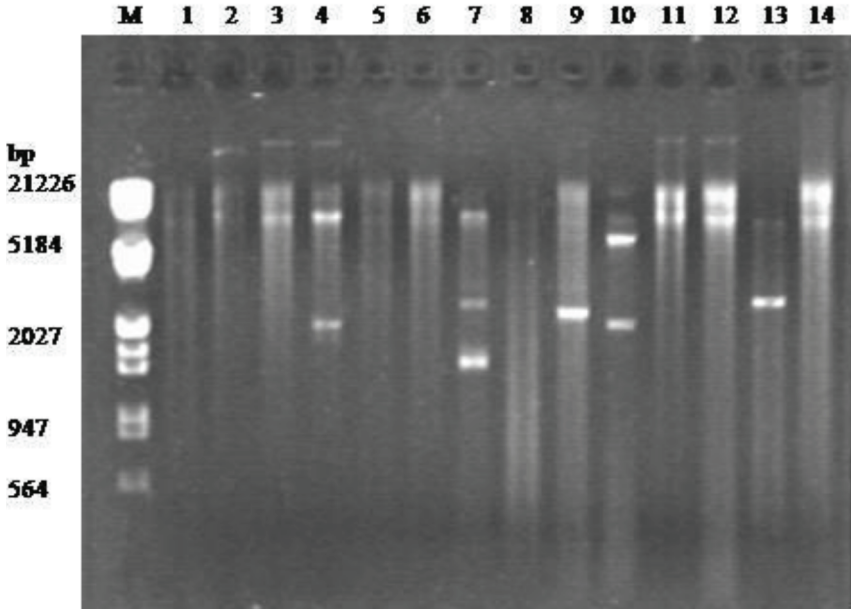

Figure 3. Bacterial plasmids showing variability in number and size. Lane M: *EcoRI* + *HindIII* DNA marker. Lanes 2, 3, 4, 7, 9–14: Visible plasmids. Lanes 1, 5, 8: No visible plasmids.

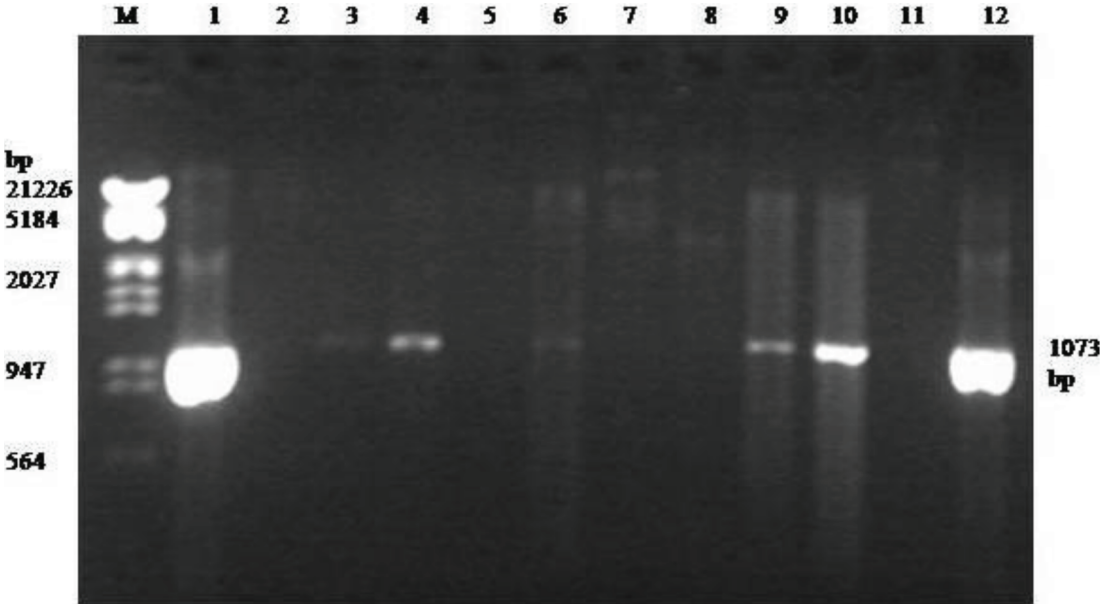

Figure 4. Amplification of full coding region of bla_{TEM} gene(1073 bp). Lane M: *EcoRI* + *HindIII* DNA marker. Lanes 1, 3, 4, 6, 9, 10: Test positive. Lanes 2, 5, 7, 8: Test negative. Lane 11: Negative control. Lane 12: Positive control.

possession of ESBL genes and Shiga toxin genes in *E. coli* isolated from healthy buffalo was reported (21). Moreover, a positive correlation between ESBL genes and other virulence genes (Shiga toxins) was recorded among *E. coli* strains isolated from diarrheic piglets in the Mizoram state of India (22).

The resistance rate of 61.6% to ceftazidime among ESBL producers in the present study was contrary to a previous study that reported a resistance rate of 4.2% to ceftazidime (17). This variation in the resistance rate of bacteria of different locations might indicate different selective pressures on the use of selectively different

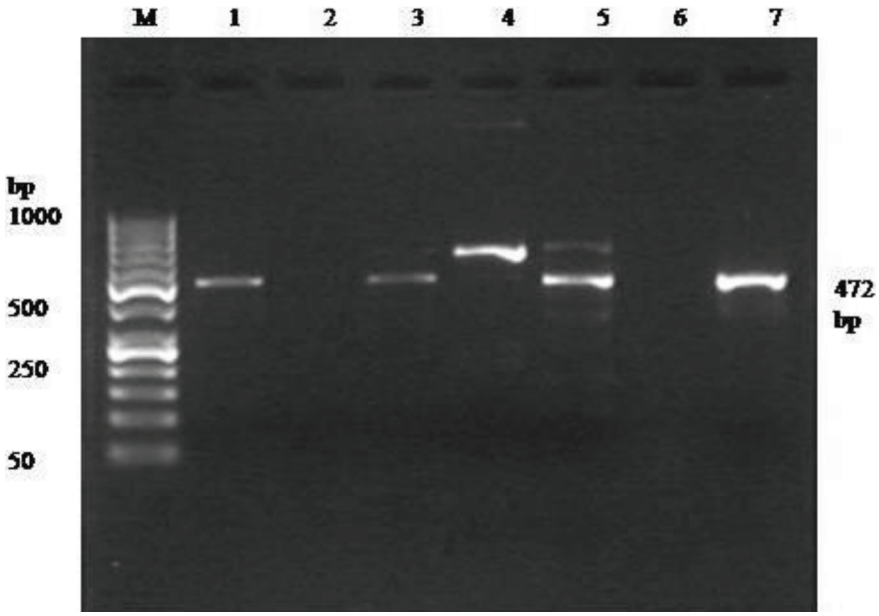

Figure 5. Amplification of *bla*$_{SHV}$ gene(472 bp). Lane M: 50-bp DNA Ladder. Lanes 1, 3, 5: Test positive. Lane 2: Test negative. Lane 4: Nonspecific. Lane 6: Negative control. Lane 7: Positive control.

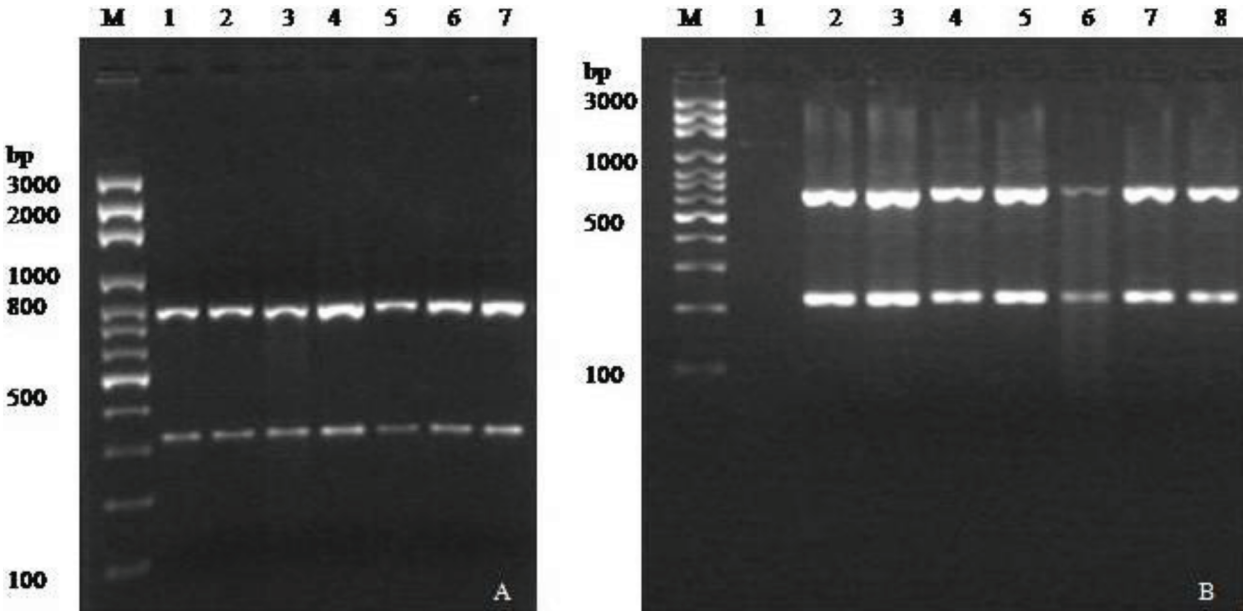

Figure 6. A) RE analysis of *bla*$_{TEM}$ by *PstI*. Lane M: M-DNA 100-bp ladder. Lanes 1–7: RE fragments (758 bp and 315 bp). B) RE analysis of *bla*$_{TEM}$ by *AvaII*. Lane M: Ladder. Lanes 2–8: RE fragments (636 bp, 222 bp, 215 bp). Lane 1: undigested band.

antibiotics. This selective use of a type of cephalosporin directly would be correlated with the resistance trend to that antibiotic, as has been reported for pigs treated with ceftiofur or cefquinome (23).

As per the CLSI (9), cephalosporins like cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), and ceftriaxone (30 µg) are used for the initial screening of ESBLs among *Klebsiella pneumoniae*, *K. oxytoca*, and *E. coli*. For all the ESBL-producing strains, the test interpretation should be reported as resistant to extended-spectrum cephalosporins including cefixime (5 µg). In the present study, too, we have recorded the bacterial isolates

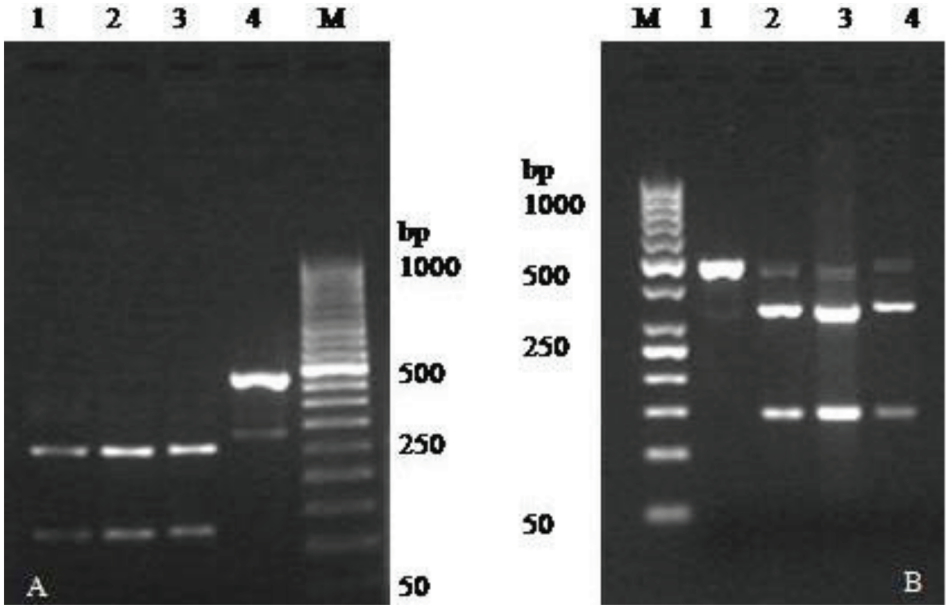

Figure 7. A) RE analysis of *bla*$_{SHV}$ by *PstI*. Lane M: 50-bp DNA ladder. Lanes 1–3: RE fragments (304 bp and 168 bp). Lane 4: Intact product (positive). B) RE analysis of *bla*$_{SHV}$ by *AvaII*. Lane M: 50-bp DNA ladder. Lane 1: Intact product (positive). Lanes 2–4: RE fragments (330 bp and 142 bp).

resistant to ceftazidime and cefixime and also observed the ESBL phenotypic test by combination disk method using ceftazidime (30 µg) and ceftazidime (30 µg)-clavulanic acid (10 µg), which is indicative of ESBL production by the bacterial isolates. These findings on ESBL screening using the phenotypic methods of the present study are consistent with previous reports (7,24). Phenotypic detection of ESBLs and carbapenemases aids in the rapid clinical diagnosis of resistant bacterial pathogens (24). Bacterial resistance to amoxicillin-clavulanic acid might be due to the expression of inhibition-resistant beta-lactamases from the isolates.

In Enterobacteriaceae, the antimicrobial resistance genes reside in plasmids. Responding to the selective pressure of antimicrobials, the plasmids, through functional expression, protect their hosts (bacteria) (25). The numbers and sizes of plasmids varied from bacteria in the present study. Moreover, many bacterial isolates shared a common plasmid pattern. Similar plasmid profiles with or without variability among Enterobacteriaceae bacteria have been reported earlier (26), thus agreeing with the findings of the present study. We also recorded larger plasmids of >21 kb in size. The ESBLs, AmpC beta-lactamases, and inhibitor-resistant derivatives of TEM and SHV acquire resistance through large plasmids (27). Furthermore, the similarity in plasmids among different isolates suggested plasmid movement between bacteria. However, a variability of plasmids among bacteria of the same taxonomical groups indicated their evolution and spread from different niches.

Molecular detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ among the bacterial isolates using PCR could be correlated with the functional expression of their resistance to the extended-spectrum cephalosporins and by a phenotypic method of ESBL screening. Coexpression of two or more resistance genes potentiates the bacterial resistance spectrum against the antimicrobials of one or more than one class. Similar coexpression of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes among *E. coli* and *Klebsiella* spp. strains was reported earlier (10), thus supporting the findings of the present study. More phenotypic positivity of antimicrobial resistance compared to the PCR-based detection of ESBL genes in the present study might be due to the presence of other probable resistance genes. Among the carbapenem-resistant isolates, the PCR negativity to the *bla*$_{KPC}$ or *bla*$_{NDM}$ gene might be due to the possibility of other carbapenemases (IMP, VIM, OXA, CMY, etc.) that could not be targeted in the present study. The production of cephalosporinase (AmpC-beta-lactamase and ESBLs have low-level carbapenemase activity) and porin loss also develops in enterobacteria resistant to carbapenems (28).

RE analyses of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes indicated the presence of common restriction sites in DNA fragments after *PstI* and *AvaII* restriction digestion. These results could be supported by a previous study (10) that reported a uniform band pattern for *bla*$_{TEM}$ and *bla*$_{SHV}$ genes after *PstI* digestion, therefore indicating a point source or a common evolutionary origin of all ESBL isolates. However, two PCR products representing the *bla*$_{SHV}$ genes indicated nucleotide

variation, which suggested that these bacteria might have acquired the *bla*$_{SHV}$ alleles from different sources.

Uniformity in nucleotide sequences of the *bla*$_{TEM}$ gene in the present study indicated a common structure of the encoded beta-lactamases. This unaltered amino acid sequence (computationally translated) of the *bla*$_{TEM}$ gene might be due to a redundancy of the genetic code. This uniformity in *bla*$_{TEM}$ gene sequences could be correlated with the findings of a previous study that reported that bacteria harbor *bla*$_{TEM-1}$ as a common *bla*$_{TEM}$ allele due to their fitness advantage in the presence of penicillins (29). Selective pressure due to prolonged consumption of particular antibiotics might be a possible factor contributing to the high frequency of the *bla*$_{SHV}$ gene subtype. The nonclonal *bla*$_{SHV}$ gene encoded common amino acid sequences except glycine (G) at codon position 130 (position 234 from the initiation codon) and glutamic acid (E) at codon position 131 (position 235 from the initiation codon) with respect to the computationally translated protein of 156 amino acids from 468 nucleotides (out of 472 nucleotides). The presence of different plasmid-mediated alleles of the beta-lactamase genes (*bla*$_{SHV}$) in the present study indicated that the plasmids might have acquired these genes possibly on transposable elements from different sources (30). An earlier study from Switzerland showed a high genetic diversity of the ESBL-harboring bacterial strains of cattle (16). The relatively high rate of occurrence of diverse ESBL-producing strains of food animals indicates that there is an established reservoir of these organisms in farm animals.

In conclusion, this study highlighted the occurrence of *bla*$_{TEM}$- and *bla*$_{SHV}$-producing coliform bacteria from cattle. The presence of ESBL-producing Enterobacteriaceae in fecal samples of farm animals poses a risk of contaminating animal food products like meat and milk. It may lead to colonization of healthy humans with ESBL-producing bacteria and thus raises urgent needs for advanced antimicrobial therapy. It is recommended that a monitoring system be established to analyze the further trends of ESBL epidemiology in order to restore the efficacy of important oxyimino cephalosporins for treating the severe infections of animals and humans. Rapid phenotypic detection of ESBLs and specific detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes by PCR is helpful in limiting the further spread of such antimicrobial resistant pathogens.

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