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Research Article

THE PORTRAYAL OF EXTENDED-SPECTRUM-β-LACTAMASES POSITIVE *KLEBSIELLA PNEUMONIAE* FROM DOMESTIC ANIMALS IN WEST BENGAL, INDIA, WITH THE EVALUATION OF THERAPEUTIC EFFICACY *IN-VITRO*

Zahid Hussain Hazarika[1], Sharmila Latchubhukta[1], Tousif Mondal[1], Siddhartha Narayan Joardar[1], Samir Dey[1], Indranil Samanta[1], Shyam Sunder Kesh[2], Kunal Batabyal[1*]

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ABSTRACT: A study investigated the antimicrobial resistance properties of *Klebsiella pneumoniae* strains positive for ESBL production, detected from raw milk and rectal swabs (N=199) of domestic animals, *viz.*, cattle (52,30), sheep (10,40), and goats (27,40) collected from a few districts of West Bengal. Seventy-seven (38.69%) *Klebsiella pneumoniae* isolates were recovered that were confirmed by PCR. The highest frequency of positive *Klebsiella pneumoniae* strains was found in rectal swab samples (50.91%) compared to milk samples (23.60%) collected from domestic animals. Thirty-five (45.45%) *K. pneumoniae* samples were isolated phenotypically as ESBL producers, whereas 68 (88.31%) isolates were positive for the presence of any one of the targeted ESBL genes. The PCR detection of the genes responsible for ESBL activity revealed the highest prevalence of the *bla*CTX-M (54.54%) gene than the other two ESBL genes, *bla*SHV (53.25%) and *bla*TEM (22.08%). All three ESBLs were isolated at a greater frequency from rectal swabs than from milk samples. In PCR, the rectal swab positive samples (48) revealed the highest prevalence of the *bla*SHV gene (64.58%) than to the milk sample isolates (20, 50%). Isolation of the *bla*CTX-M gene (75%) from the milk sample isolates was more than that of the rectal swab isolates (56.25%), while *bla*TEM was isolated from both the rectal swab and milk sample 25%. Antibiogram of all the ESBL-producing *Klebsiella pneumoniae* isolates (n = 68) against 10 antibiotics showed 100% resistance towards ticarcillin/clavulanic acid and 80-90% resistance towards enrofloxacin, amoxicillin, and cefixime. While these bacteria were highly sensitive (100%) to imipenem EDTA, doxycycline, and other antibiotics like tetracycline, chloramphenicol, gentamicin, but co-trimoxazole showed maximum overall sensitivity (60-88%).

Keywords: Antibiogram, Cattle, ESBL, Goats, *Klebsiella pneumoniae*, Milk, Sheep.

INTRODUCTION

Being an agriculture-based country, 65 to 70% of India's total population depends on agriculture and farming. A significant population of Indian farmers (around 20.5 million) relies on livestock farming for their daily bread, of which 16% are small farm households, and 14% are rural households [1]. Bovines contribute a major part to this. India ranks first, contributing to 23% of global milk production [2]. Again, India possesses 148.88 million goats, *i.e*., the 2nd position in the world and 3rd position in sheep (74.26 million). The state of West Bengal possesses a major share of the country's livestock population, with 37.4 million, of which the Cattle population is about 19 million. According to the census, the state accounts for a 16.28 million goat population [3].

The performance of this livestock population mostly depends on animal health and other factors. Animals can suffer from several bacterial, viral, and parasitic infections, leading to a reduction in their performance. A few infectious diseases may be zoonotic and can spread from animals to humans. Antibiotic-resistant Gram-

[1]*Department of Veterinary Microbiology,* [2]*Dept. of Veterinary Clinical Complex, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences Kolkata-700 037, India.*
**Corresponding author. e-mail: drkb.micro@gmail.com*

negative bacteria like *Klebsiella pneumoniae* can cause different types of infections in bovines like pneumonia, enteritis, mastitis, and other septicaemic conditions, drastically reducing their production performance [4].

A Gram-negative bacterium, *Klebsiella pneumoniae*, is an opportunistic pathogen and member of the *Enterobacteriaceae* family, commonly found in nature and grows in the gastrointestinal tract. tracts of healthy human beings and animals [5]. It causes pneumonia, cystitis, pyelonephritis, septicaemia, and pyogenic liver abscess-like diseases in humans and other animals [6]. Bovine mastitis/sub-clinical mastitis leads to a significant decrease in milk yield and quality, affecting the rural economy, caused by *Klebsiella* spp. along with other significant pathogens [7, 8].

Small ruminants like goats and sheep are also susceptible to *Klebsiella pneumoniae* infection, which causes pneumonia and septicaemia, which can result in occasional death due to unsanitary conditions [9]. Furthermore, emerging antibiotic resistance in some *Klebsiella pneumoniae* strains (with ESBL positivity) has worsened the situation. However, in West Bengal, a lower prevalence of ESBL-producing *Klebsiella* in goats (6.2%) and in sheep (10%) was reported by Banerjee et al. (10). This was because of a lack of exposure to cephalosporin. The prevalence of ESBL-positive Klebsiellae in domesticated animals, viz., sheep and goats, may be linked to exposure to the environmental resistance gene bank, even if not being exposed to third-generation cephalosporins [10].

Bacteria, Gram-negative in nature, mostly generate enzymes like extended-spectrum beta-lactamases (ESBLs) that can hydrolyze various antibiotics with β-lactam rings in their structure, thereby showing resistance to penicillins, beta-lactam antibiotics, third and fourth-generation cephalosporins, and other antibiotics [11]. These genes generally remain in the plasmids and can easily be transferred from one bacterium to another. Anti-microbial resistance towards β-lactam antibiotics has been a problem throughout the history of their use [12]. Bacteria, namely *Escherichia coli* and *K. pneumoniae,* produce different ESBL-producing genes like CTX-M, TEM, and SHV [13, 14, 15]. With these resistance factors, different varieties of endotoxins / O-lipopolysaccharide (LPS), capsular antigens (K), adherence factors, and siderophores are liable for the pathogenicity of *K. pneumoniae* strains [16, 17]. With this background, the present study is designed to detect antibiotic-resistant *Klebsiella pneumoniae* strains from the milk samples and rectal swabs from different domestic animals like cattle, sheep, and goats from a few districts of West Bengal with their *in-vitro* antibiogram.

MATERIAL AND METHODS

Approval of the institutional biosafety committee

As there is no use of any laboratory or domestic animals for experimental research, no institutional animal ethics committee clearance is required. But the research work got approval from the institutional biosafety committee (Order no. FVAS/Micro-IBSC/04/22-23, dt., 05/12/2022) for handling potentially zoonotic pathogens as per standard methods in compliance with national guidelines.

Details of sample collection

One hundred ninety-nine samples (89 milk samples, 110 rectal swabs) were collected from apparently healthy indigenous and crossbreed domestic animals (cattle, sheep, and goats) from unorganized dairy farmers/animal owners from June to November 2022, from the following districts of West Bengal, namely Nadia (Mohanpur), Purba Barddhaman (Ayushgram), Birbhum (Siuri), and Purba Medinipur (Kanthi). No organized dairy farm was targeted. The animals were examined and found to be healthy with no history of detectable illness or symptoms of mastitis or other infections. The sample size was small due to a lack of funds and time for the study. The milk samples were instantly and directly collected from the udder, in sterile vials with sterilized peptone water (HiMedia, India). The rectal swab samples were collected aseptically with sterile cotton swabs (HiMedia, India) in separate vials with sterile peptone water (HiMedia, India) [10]. All collected samples were kept in a clean sample collection flask under the ice pads' cover and were brought to the laboratory for further processing via the shortest route.

Detection of *Klebsiella* spp. from collected samples

All the collected samples were enriched at 37°C for 18 hrs in peptone water aseptically. Enriched samples were streaked onto Hi-chrome *Klebsiella* selective agar (HiMedia, India) plates and incubated overnight at 37°C. The characteristic magenta-coloured mucoid colonies were selected and preserved on nutrient agar (HiMedia, India) slants, subsequently for further characterization (morphological and biochemical ones) [18].

Pinpointing of the *Klebsiella* spp. isolates

All the isolates were examined for morphology after Gram's method of staining and biochemical characterization (Catalase, Oxidase, and IMViC tests) was done as per methods described by Quinn *et al.* [18] and Edward and Ewing [19].

Molecular confirmation of the *Klebsiella pneumoniae* isolates

All tentatively confirmed *Klebsiella* spp. isolates were considered for molecular confirmation by PCR.

Extraction of bacterial DNA

All isolates were enriched again overnight, in 2ml Nutrient broth (HiMedia, India) at 37°C. Organisms were pelleted after centrifugation at 8000 rpm for 10 min. The obtained bacterial pellet was resuspended in 150µl of nuclease-free water (Hi-media, India) and lysed by boiling for 10 min in a water bath and then chilled immediately. The template DNA for PCR was considered as the supernatant after removing the cell debris by centrifugation at 2000 rpm for 5 min [20].

Confirmation of *K. pneumoniae* isolates by detection of the *16S rRNA* gene in PCR

The positive *Klebsiella* spp. strains were again checked for the presence of the *Klebsiella pneumoniae*-specific gene as described by Liu *et al.* [21]. The amplified products were visualized after electrophoresis with the help of a gel documentation system (Bio-Rad). The positive control was supplied by the department itself. Table 1 describes all the details about the primers, conditions, and the length of PCR amplification products.

Phenotypic detection of ESBL positivity in *Klebsiella pneumoniae* isolates

The confirmed *K. pneumoniae* strains were evaluated using the double disc synergy assay (DDSA) using Cefotaxime (30µg, HiMedia, India) and Ceftazidime (30µg, HiMedia, India) discs with and without clavulanate (10µg, HiMedia, India) [22] for the *in-vitro* expression of the ESBL genes present in those, in compliance with guidelines recommended by Clinical and Laboratory Standards Institute, 2022 data [23] and El-Hariri *et al.* [24]. The standard distance was 20 mm between the centres of the discs for each set. After overnight incubation at 37°C, the plates were examined to check phenotypic expression of ESBL-producing genes in the strains, which was confirmed by observing a difference of ≥ 5mm between the zones of inhibition in the presence and absence of clavulanate discs, as per CLSI 2022 [23].

Molecular detection of the ESBL genes in *Klebsiella pneumoniae* strains

The *Klebsiella pneumoniae* isolates were assessed by PCR to detect major ESBL-producing genes, namely, *bla*SHV, *bla*TEM, and *bla*CTX-M. Primers and the predicted length of the PCR amplification products are detailed in Table 1. One *Klebsiella pneumoniae* isolate (MTCC 4030) was used as the positive control in these studies. All negative controls were kept as blanks/ uninoculated. All primers used in this study were previously validated for confirmation of their amplicon size.

Spotting of the CTX-M gene in *K. pneumoniae* isolates by PCR

The PCR detection of the *bla*CTX-M gene in *K. pneumoniae* isolates was done following the method described by Weill *et al.* [25], adding slight modifications. The amplified products were visualized after electrophoresis using a gel doc. system (Bio-Rad).

Detection of the SHV gene in K. pneumoniae isolates by PCR

PCR was done to detect the *bla*SHV genes in *Klebsiella pneumoniae* strains following the activities of Cao *et al.* [26]. PCR products were visualized by electrophoresis, with the help of a gel documentation system (Bio-Rad).

PCR confirmation of the TEM gene in *Klebsiella pneumoniae* isolates

The *bla*TEM gene was targeted in the *K. pneumoniae* strains after the protocol of Weill *et al.* [25] with some modifications. The amplified products were detected in a 2% agarose gel electrophoresis (W/V) containing ethidium bromide (0.5µg/ml) [SRL, India] by gel doc. system (Bio-Rad). Sequencing of the detected genes/representative amplicons and their submissions to GenBank were incomplete due to insufficient time and funds.

Antibiotic efficacy evaluation of ESBL-positive *K. pneumoniae* isolates

In-vitro testing of the sensitivity and resistance patterns of all the ESBL-producing *K. pneumoniae* isolates was done by the disc diffusion method [22] using different antibiotics to assess their therapeutic efficacy. The antibiotic discs used were amoxicillin, gentamicin, doxycycline, chloramphenicol, co-trimoxazole, imipenem EDTA, enrofloxacin, ticarcillin/ clavulanic acid, cefixime, and tetracycline (Hi-Media). The antibiotics were selected as the commonly used drugs in both veterinary and medical practices, and as per their efficacy reports against the Gram-negative bacilli in particular. The susceptibility criteria were applied as per the revised data sheet of CLSI, 2022 [23] M100 document.

RESULTS AND DISCUSSION

Isolation and confirmation of the *Klebsiella* spp. isolates

Eighty-nine milk and 110 rectal swab samples were assembled from domestic animals, including dairy cows/ cattle, goats, and sheep, for this research work. One hundred and six (53.26%) *Klebsiella* spp. Strains were isolated in this study, of which 38 (42.69%) were from 89 milk samples and 68 (61.81%) from rectal swabs of all the animals. All the isolates were tentatively detected as *Klebsiella* spp. based on their morphological and colony characteristics. All showed typical purple magenta-coloured colonies on Klebsiella selective agar plates and appeared as Gram-negative, encapsulated, rod-shaped bacilli in morphological examinations. The highest prevalence of *Klebsiella* spp. was seen in Goat milk (66.67%) (18/27), followed by Sheep 50% (5/10) and Cow milk 28.84% (15/52) [Table 2]. No seasonal variation was studied here. The pervasiveness of *Klebsiella* spp. from goat milk was higher, possibly due to the variation in sampling and other environmental / geographical factors. Again, rectal swab samples of cattle showed the highest prevalence (83.33%) followed by the other two animals. In a similar study, on randomly collected raw milk samples in Egypt, Tartor *et al*. [27] found 36% of isolates to be positive for *Klebsiella pneumoniae,* which is somewhat similar to the current report. In another study, Badri *et al*. [28] reported a

Table 1. List of Primers used in this study with product sizes and PCR conditions.

Sl. No.	Target Genes amplified	Primer sequences (5 PCR Conditions	Product size (bp)	Reference(s)
01	*Klebsiella pneumoniae*	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC The PCR reaction was performed with 25µl final mix. containing 1.5 mM $MgCl_2$, 1 µm of each primer, 0.1 mM each dNTP, 1-unit Taq DNA polymerase, plus 5 µl DNA template. The samples were subjected to 35 cycles each of the cycles consisted of 94°C for 30s, 57°C for 20s, 72°C for 20s, and a final extension of 10 mins at 72°C.	130	21
02	bla_{CTX-M}	F: CAATGTGCAGCACCAAGTAA R: CGCGATATATCGTTGGTGGTTGGTG The reaction mixture (25µl) contains pure DNA templates (5µl), 2 primers (50pmole each), dNTPs (200mM), Taq DNA polymerase [1U] (Promega, USA), $MgC1_2$ (2mM) and 10% dimethyl sulfoxide. The reaction mixtures were subjected to PCR amplifications which included a series of cycling conditions of initial denaturation at 94°C for 10mins, then 30s of denaturation at 94°C, 30s of annealing at 53°C and 1 min of extension at 72°C for 35 cycles, and 10mins of final extension at 72°C.	540	25
03	bla_{SHV}	F: TTATCTCCCTGTTAGCCACC R: GATTTGCTGATTTCGCTCGG A 25µl reaction mixture contains 5µl DNA templets, 2mM $MgCl_2$, 200µM dNTPs, 1U Taq DNA polymerase (Promega, USA), 50 picomoles of each primer, and 10% dimethyl sulfoxide (DMSO) and PCR was done in Bio-Rad PCR systems following these conditions: 10 mins of initial denaturation at 94°C, followed by final denaturation at 94°C for 45secs., 1 min annealing at 52°C, and 1 min extension at 72°C for 35 cycles, and 10 mins final extension at 72°C.	792	26
04	bla_{TEM}	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC The final volume of the reaction mixture was 25µl consisting of test bacterial DNA template (5µl), both the primers (@50pmol), and deoxynucleoside triphosphate (200M), 1 U Taq DNA polymerase (Promega, USA), 2mM $MgC1_2$ and dimethyl sulfoxide (10%). The reaction mixtures were subjected up to 10mins of denaturation at 94°C (one cycle); 30s of denaturation at 94°C, 1min of annealing at 53°C and 1min of extension at 72°C (35 cycles), followed by 10mins of extension at 72°C.	1080	25

higher frequency of *Klebsiella pneumoniae* isolates (62%) in raw cow milk samples from Al Jazirah, Sudan, while in North-Eastern Indian states, a lower prevalence rate of *Klebsiella pneumoniae* (10.8%) isolates from healthy cow milk was revealed by Koovapra *et al*. [29]. Still, the isolation rate was higher in the case of Sub-clinical mastitis-infected milk (68.5%).

In mastitic goats, the prevalence of *K. pneumoniae* was approximately. 9.6% as reported by Satpathy *et al.* [30], which is quite less than the present findings, may be due to geographical variation. Montso *et al.* [31] reported a lower prevalence of *Klebsiella* spp. from cattle rectal swabs (32%). A lower prevalence rate of *K. pneumoniae* from goat rectal swabs (5%) was also reported by Sghaier *et al.* [32]. In West Bengal, Banerjee *et al.* [10] found similar results for *Klebsiella* spp. isolates from sheep and goats as 10% and 6.2%, respectively.

Biochemical characterization of all these tentatively positive *Klebsiella* spp. isolates (n=106) showed standard results such as positive to Catalase, Citrate utilization tests, & Voges-Proskauer (VP), and negative to Indole, Oxidase, and Methyl red tests. These results are also supported by Banerjee *et al*. [10], Quinn *et al.* [18], and Samanta [33].

Table 2. Sample-wise prevalence of *K. pneumoniae* isolates.

Types of samples	Nos. of *Klebsiella* spp. isolated (%)	Nos. of *K. pneumoniae* isolates confirmed by PCR (%)	Nos. of *K. pneumoniae* isolates phenotypically ESBL positive	Nos. of *K. pneumoniae* isolates genotypically ESBL positive
Cow Milk (52)	15 (28.84%)	7 (13.46%)	4 (57.14%)	7 (100%)
Sheep Milk (10)	5 (50%)	4 (40%)	1 (25%)	3 (75%)
Goat Milk (27)	18 (66.67%)	10 (37.04%)	4 (40%)	10 (100%)
Cattle Rectal Swabs (30)	25 (83.33%)	22 (73.33%)	10 (45.45%)	18 (81.82%)
Sheep Rectal Swabs (40)	23 (57.50%)	18 (45%)	7 (38.89%)	16 (88.89%)
Goat Rectal Swabs (40)	20 (50%)	16 (40%)	9 (56.25%)	14 (87.5%)
Total (199)	106 (53.27%)	77 (38.69%)	35 (45.45%)	68 (88.31%)

Confirmation of *K. pneumoniae* strains by PCR detection of the *16S rRNA* gene

Among the *Klebsiella* spp. isolates detected morphologically and biochemically, only 77 (72.64%) were affirmed to be *Klebsiella pneumoniae* having the *16S rRNA* gene (130bp) specific for the species (Table 2, Fig. 1). Cattle rectal swab (73.33%) and goat milk (37.04%) samples showed a higher prevalence of the bacterium that the other sources (Table 2). The differences in prevalence of *K. pneumoniae* in milk vs. swabs are significant with a p-value < 0.05 after a Chi-square test. Samples collected from the Purba Barddhaman district showed the highest prevalence (48.57%) of *K. pneumoniae* isolates, followed by other districts, viz. Nadia (38.33%), Birbhum (33.33%), and Purba Medinipur (37.5%). These prevalence reports were not significant at p-value < .05, but can point out differences in the endemic nature of the bacterium over various places. Seru *et al.* [34] showed a lower positivity of *Klebsiella pneumoniae* (4%) in raw cow milk samples from West Bengal, showing the possible risk of public health hazards, which is quite lower than the present findings. Again, in their study in Tunisia, Zadoks *et al.* [35] reported an almost similar prevalence of *Klebsiella pneumoniae* (84%) from the rectal swabs of cows. The present report clearly denotes the potential transmission possibilities of this bacterium to human beings via milk and their environmental reservoirs, the cattle population. The significant presence of this bacterium in bovine milk and faeces can easily be transmitted to humans through various pathways, including person-to-person contact, environmental contamination, or reservoirs like soil, food, contaminated water, and medical equipment, and healthcare settings [36].

Phenotypic expression of extended spectrum β-Lactamase (ESBL) genes in the under study *Klebsiella pneumoniae* isolates

Based on a Double Disc Synergy Assay, out of 77 *Klebsiella pneumoniae* isolates, 35 (45.45%) showed *in-vitro* expression of ESBL activity (Table 2, Fig. 2). A report by Montso *et al.* [31] showed the presence of 53.1% *K. pneumoniae* isolates to be positive for

Table 3. Species-wise positivity of 3 ESBL genes in *Klebsiella pneumoniae* isolates.

Types of hosts	No. of bacteria tested	No. of isolates with *bla*CTX-M gene	No. of *bla*SHV positive isolates	No. of *bla*TEM positive isolates
Cattle	29	18 (62.06%)	17 (58.62%)	06 (20.68%)
Sheep	22	9 (40.90%)	8 (36.36%)	07 (31.81%)
Goat	26	15 (57.69%)	16 (61.54%)	04 (15.38%)
Total	77	42 (54.54%)	41 (53.25%)	17 (22.08%)

Table 4. Sample-wise frequency of ESBL genes in *Klebsiella pneumoniae* isolates.

Types of species	Positive Milk isolates	Positive Rectal Swab isolates	Total	%
CTX-M	7	10	17	25.0
SHV	3	13	16	23.53
TEM	1	5	6	8.82
CTX-M+SHV	5	13	18	26.47
CTX-M+TEM	2	2	4	5.88
SHV+TEM	1	3	4	5.88
All 3	1	2	3	4.41
Total	20	48	68	88.31

Table 5. Sensitivity and Inefficacy patterns of the ESBL-yielding *Klebsiella pneumoniae* strains (n = 68).

Sl. No.	Used Antibiotics (Concentration)	Sensitive (%)	Intermediate (%)	Resistant (%)
01	Amoxicillin (10µg)	12.06	3.44	84.48
02	Gentamicin (10µg)	82.75	12.06	5.17
03	Doxycycline (30 µg)	100	00	00
04	Chloramphenicol (25µg)	87.93	6.89	5.17
05	Co-trimoxazole (25 µg)	70.68	00	29.31
06	Imipenem EDTA (10 µg)	100	00	00
07	Enrofloxacin (5 µg)	8.62	5.17	86.20
08	Ticarcillin/Clavulanic acid (70/10 µg)	00	00	100
09	Cefixime (10 µg)	13.79	3.44	82.75
10	Tetracycline (30 µg)	60.34	22.41	17.24

phenotypic ESBL production after screening 151 cattle samples, including 96 samples of raw beef and 55 samples of bovine faeces. Gholipour *et al.* [37] reported the phenotypical occurrence of ESBL in the isolates was as follows: 107 (43.67%) out of 245 *E. coli* and 21 (38.18%) out of 55 *K. pneumoniae* strains in their study, which was less than the present report. Again, Chaisaeng *et al.* [38] reported that they tested 70 *K. pneumoniae* isolates by double disk diffusion assays, to reveal that 54 out of the 70 isolates (77.1%) were positive for phenotypical ESBL production. These higher incidences might be due to geographical and sampling differences.

PCR proofing of ESBL-producing genes in *Klebsiella pneumoniae* strains

All the *K. pneumoniae* isolates (including the phenotypic test-positive ones) were screened by PCR for the presence of any one of the three ESBL-producing genes to reveal the 68 positive isolates with the following gene distributions :

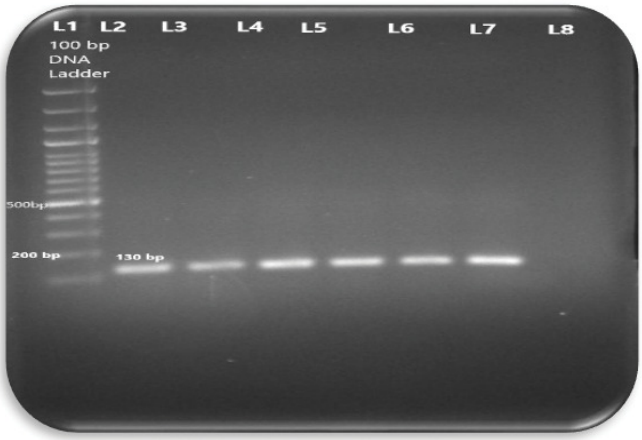

Fig. 1. Agarose gel electrophoresis showing the confirmation of *Klebsiella pneumoniae* strains with the detection of the specific gene (130 bp) [L1: 100 bp DNA Ladder, L2: Positive control, L3-L7: Test samples, L8: Uninoculated Negative control]

Fig. 2. Double disc Synergy Assay showing the phenotypic expression of ESBL-producing genes in the *Klebsiella pneumoniae* isolates.

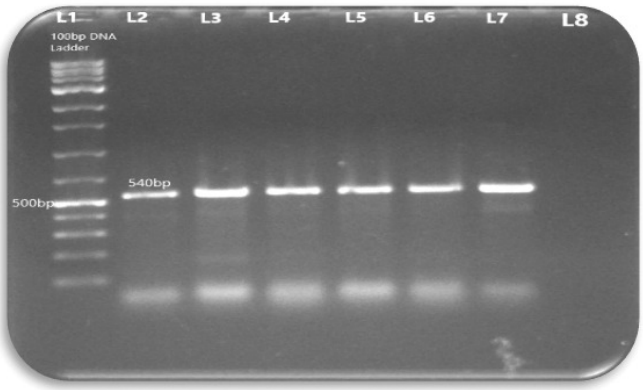

Fig. 3. Agarose gel electrophoresis showing the proof of the *bla*CTX-M gene (540 bp) positivity in *K. pneumoniae* strains [L1: 100 bp DNA Ladder, L2: Positive control, L3-L7: Test samples, L8: Uninoculated Negative control].

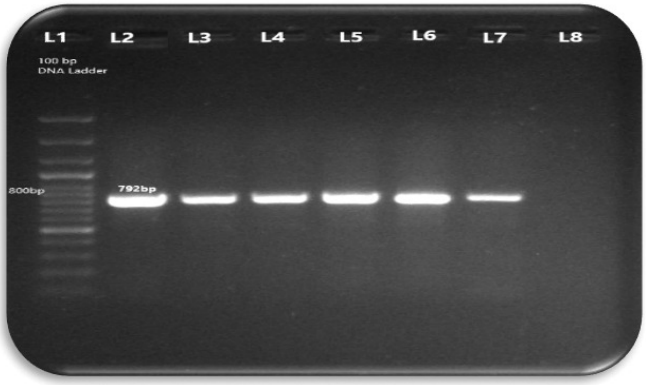

Fig. 4: Gel electrophoresis showing the evidence of the *bla*SHV gene (792 bp) in *K. pneumoniae* strains [L1: 100 bp DNA Ladder, L2: Positive control, L3-L7: Test samples, L8: Uninoculated Negative control].

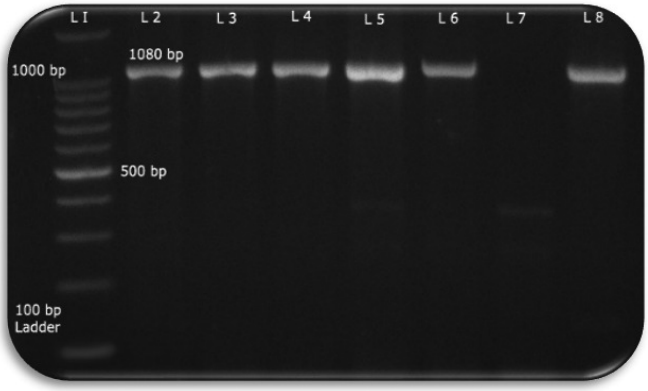

Fig. 5. Figure of an agarose gel showing the presence of *bla*TEM gene (1080 bp) in *K. pneumoniae* isolates [L1: 100 bp DNA Ladder, L2-L6: Test samples, L7: Uninoculated Negative control, L8: Positive control].

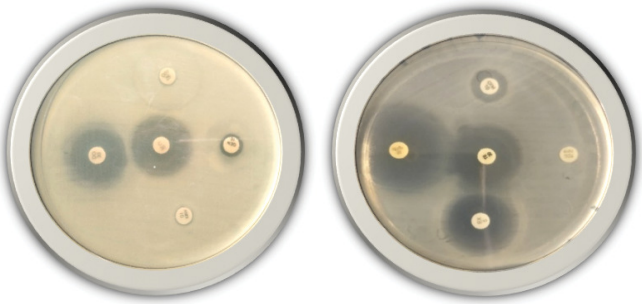

Fig. 6. **Fig. 7.**

Fig. 6. and Fig. 7. *In-vitro* sensitivity and inefficacy pattern of ESBL-generating *K. pneumoniae* strains.

PCR recognition of the *bla*CTX-M gene in *Klebsiella pneumoniae* isolates

A total of 42 (54.54%) *K. pneumoniae* isolates were positive to have the *bla*CTX-M gene (Fig. 3, Table 3) with the PCR-amplified product 540 bp. Sample-wise analysis showed 16.85% *bla*CTX-M positivity in milk sample isolates, while isolates from rectal swab samples showed higher positivity of 24.55%, which was not found to be significant at $p < 0.05$. Three (4.41%) isolates showed the presence of all three genes in this study (Table 4).

Among the three species, the highest incidence rate of the CTX-M gene was found in cow strains (62.06%), followed by the goat and sheep strains (Table 3). In China, Zhang *et al.* [39] detected 90.8% *bla*CTX-M genes from 184 ESBL-producing *Klebsiella pneumoniae* isolates, followed by 16.84% *bla*SHV and 19.02% *bla*AmpC genes, which is quite higher than the present report. Banerjee *et al.* [10] showed a higher prevalence of *bla*CTX-M-positive *Klebsiella pneumoniae* (70.94%) isolated from rectal swabs of dogs, sheep, goats, and cats, but Cabral *et al.* [40] found 62.5% *bla*CTX-M-2 isolates for *Klebsiella pneumoniae* from hospital-related samples, which almost matches the present findings. Almost such significant positivity of the CTX-M-1 gene (61%) was reported in a study by Badri *et al.* [28] from raw milk samples from *K. pneumoniae* strains. Thus, the present report stands confirmed.

Confirmation of the evidence of the *bla*SHV gene in *K. pneumoniae* isolates

Forty-one (53.25%) *Klebsiella pneumoniae* isolates were revealed to have the *bla*SHV gene with a PCR-amplified product of 792 bp (Fig. 4, Table 3). Sample-wise analysis showed that the data of incidence was higher in rectal swabs (28.18%) than in the milk samples (11.23%). The result is found to be significant at $p < 0.05$. *Klebsiella pneumoniae* isolates from goats showed the highest prevalence or incidence rate (61.54%) of the *bla*SHV gene, followed by cattle (58.62%) and sheep (36.36%) isolates. Significant evidence of the SHV gene (34.30%) in *K. pneumoniae* isolates of cattle faeces origin and raw beef samples was shown by Montso *et al.* [31], which is quite less than the present report. Das et al. [41] reported a higher presence of the *bla*SHV genes (77.97%) in *K. pneumoniae* from milk, faecal, and meat samples of animal origin. Ejaz et al. [42] recently reported *Klebsiella pneumoniae* strains from cattle with 26.1% positivity in *bla*TEM, 73.9% in *bla*CTX-M, and 14.2% in *bla*SHV genes, which almost supports the present study reporting the existence of the ESBL genes in Klebsiellae samples.

Prevalence of the *bla*TEM genes in *K. pneumoniae* isolates – PCR confirmation

Around 17 *K. pneumoniae* strains (22.08%) had the *bla*TEM gene (1080bp) as found in this study (Fig. 5, Table 4). Sample-wise analysis of the samples showed that isolates from the rectal swabs had having higher prevalence (10.91%) of the gene in comparison to the milk sample isolates (5.61%), but this result is not detected to be significant at $p < 0.05$. Again, among the three hosts, the incidence rate of the *bla*TEM gene was the highest in sheep isolates (31.81%), followed by cattle and goat isolates. Scientists like Iweriebor *et al.* [43] studied *K. pneumoniae* strains from animal faecal samples (n=400) and reported multiple antibiotic resistance genes, with 27% positivity of the *bla*TEM gene with other genes. Reports of Badri *et al.* [28] (*bla*TEM gene 16.04%) and Kpoda *et al.* [44] reported proof of different ESBL genes like *bla*TEM (24.66%) and others in Gram-negative bacteria, which almost matches the present report and thus stands confirmed.

***In-vitro* antibiotic sensitivity testing of ESBL-producing *K. pneumoniae* isolates**

The antibiogram of ESBL-positive *Klebsiella pneumoniae* isolates showed that all *K. pneumoniae* strains tested were fully resistant to ticarcillin/clavulanic acid, and most antibiotics showed insensitivity towards the bacteria. The ESBL-producing *Klebsiella pneumoniae* isolates showed 86.20%, 84.48%, and 82.75% resistance against enrofloxacin, amoxicillin, and cefixime, respectively. Doxycycline and imipenem were two antibiotics that showed 100% sensitivity, whereas chloramphenicol, gentamicin, co-trimoxazole, & tetracycline showed 87.93%, 82.75%, 70.68%, and 60.34% sensitivity, respectively, towards ESBL *K. pneumoniae* (Fig. 6 & 7, Table 5). This report indicates the possible control measures for this bacterium *in vitro* using these few antibiotics, namely doxycycline, imipenem, gentamicin, co-trimoxazole, etc.

In a similar study, Bobbadi *et al.* [45] revealed the presence of 89 *K. pneumoniae* isolates capable of ESBL production, from livestock and livestock product samples, which were highly sensitive towards imipenem (100%) and gentamicin (95.05%). In another study, Yang *et al.* [46] isolated 66 *K. pneumoniae* from milk samples and found 100% resistance against amoxicillin, ampicillin followed by tetracycline (21.21%), chloramphenicol (13.64%), gentamicin (12.12%),

tobramycin (12.12%), cefuroxime (4.55%), ceftazidime and piperacillin (both 3.03%), and cefoperazone (1.52%) which were comparatively similar to the present study.

Das *et al.* [41] in a study observed high resistance level by ESBL-producing *K. pneumoniae* towards ceftizoxime (69.49%) followed by co-trimoxazole (25.73%), chloramphenicol (16.95%), ciprofloxacin (25.42%), tetracycline (23.73%), piperacillin-tazobactam (5.08%), and gentamicin (3.39%) while imipenem and meropenem were effective against 100% isolates.

CONCLUSION

The prevalence of *Klebsiella pneumoniae* strains (38.69%) reported here was quite significant from a zoonotic point of view. The higher (50.9%) frequency in rectal swabs than in the milk samples (23.6%) was reported from the samples studied here. Again, the sheep samples revealed the highest (44%) positivity of *K. pneumoniae* followed by goats (38.8%), and cattle (35.4%) samples. A total of 35 (45.5%) isolates showed the phenotypic expression of ESBL positivity in this report. In the molecular screening for the ESBL genes, the bla_{SHV} gene (53.24%) was the highest, followed by the $bla_{CTX\text{-}M}$ (49.35%) and bla_{TEM} (23.37%) genes. A significantly alarming level (@80-100%) of ineffectiveness was shown by these ESBL-positive *Klebsiella pneumoniae* isolates to antibiotics like ticarcillin/clavulanic acid, enrofloxacin, and cefixime. Imipenem, EDTA, tetracycline, chloramphenicol, and gentamicin were sensitive against these ESBL-positive *Klebsiella pneumoniae* isolates.

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