Detection of genetic determinants of virulence and ESBL genes in *Klebsiella pneumoniae* isolates of small ruminants

Sravani G[1], Deepika Kumari G[2], Bindu Kiranmayi Ch[3] and Anand Kumar P[4]

[1]Department of Veterinary Microbiology, [3]Department of Veterinary Public health and Epidemiology, [4]Department of Veterinary Microbiology,NTRCVSc., Gannavaram, Krishna district, Andhra Pradesh 521102 [2]Department of Veterinary Microbiology, CVSc., Garividi, Vizianagaram, district, Andhra Pradesh, 535101
Corresponding author email: deepu.angrau@gmail.com

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Abstract

K. pneumoniae is a Gram-negative bacterium capable of causing severe respiratory and gastrointestinal diseases, leading to significant morbidity and mortality within livestock populations. Investigating the (Extended Spectrum Beta Lactamases) ESBL's and virulence genes helps to inform effective treatment protocols and curb the spread of resistant strains, which pose a threat not only to animal health but potentially to public health through zoonotic transmission pathways. A total of one hundred and four (n=104) samples were collected, from suspected pneumonic (n=51) and enteric cases (n=53). Preliminarily, cultural and biochemical tests were carried out for the isolation of *K. pneumoniae* and molecular tests like Polymerase Chain Reaction (PCR) using species-specific 16S rRNA primers that produced an amplicon product size of 108 bp and detected 42 (40.38%) isolates of *K. pneumoniae* from the suspected cases. Out of the 42 isolates, 13 (30.9%) isolates were positive for the presence of bla_{TEM} ESBL gene and 34 (80.9%) isolates confirmed virulence *fimH* gene with an amplicon size of 688 bp. These results highlighted the presence of ESBL and virulence genes in the *Klebsiella* isolates of small ruminants.

Keywords: ESBL genes**,** *Klebsiella pneumonia,* Small ruminants, Virulence gene

Introduction

As per One Health approach, human health is related to animal health and environment, so continuous surveillance and monitoring should be carried out in all the three areas in order to check the antimicrobial resistance. Uncontrolled antimicrobial use and abuse in human, animal, and environmental sectors and the spread of resistant bacteria and resistance determinants are the key drivers of antimicrobial resistance across the globe (McEwen and Collignon 2018). *Klebsiella pneumoniae* is a Gram-negative bacterium and an opportunistic pathogen observed a high steep as an antimicrobial resistant organism in the last two decades (Ciresa *et al.,*2024; Harish *et al.,* 2026). Its adaptability to different environments and ability to evade immune defenses make it a significant health concern, leading to severe infections that may manifest with respiratory symptoms or gastrointestinal disturbances (Paczosa *et al.,* 2016).

Due to overuse or misuse of antibiotics in sheep and goat development of antibiotic resistant strains of *Klebsiella*, making infections harder to treat. The domestic animals affected with *Klebsiella* pathogen may represent a source of pathogenic and multi-drug resistant microorganisms to humans. This causes a harmful impact on humans by making the infections untreatable, prolonged illness, longer period of stay in hospitals and finally high mortality could be observed. Virulence factors are closely related to the ability for bio-film formation. The bacteria that survive the selection pressure turn to be resistant and can also pass its AMR genes to its progeny or to bacteria within same or related species thus imparts huge cost to the individual apart from possessing significant zoonotic threat.

Klebsiella has become resistant to a wide range of antibiotics including penicillin by acquiring beta-lactamase enzyme leading to emergence of Extended Spectrum beta-lactamase (ESBL) producers. The uncontrolled use of carbapenems to treat ESBL has led to resistance against it (Yigit *et al*., 2001). Carbapenem class of antibiotics is considered as one of the last resources for treating serious bacterial infections and its resistance in Gram-negative bacteria is very high (Gandra *et al*., 2016). Carbapenem resistant bacterial infections are being treated with colistin but in the due course of time, emergence of colistin resistant bacteria has also been seen in India (Kaur *et al*., 2017). These are also successful environmental organisms, allocated with their intrinsic resistance to antimicrobial drugs, adaptability to a variety of niches and ability to acquire resistance mechanisms rapidly (Arzanlou *et al*., 2017).

Klebsiella is one of the members of ESKAPE pathogen and is highly virulent and multidrug - resistant pathogen, keeping in view of its severity in causing the affections in sheep the primary objective of this study was to analyze the extent of ESBL genes and virulence genes present in the *Klebsiella* isolates of small ruminants and mitigate necessary precautionary measures to curb the antimicrobial resistance caused by *Klebsiella* spp.

Materials and Methods

Sheep and goat exhibiting clinical signs of respiratory and gastrointestinal like dyspnoea, coughing, mucus logged in nares, open mouth breathing and enteritis, respectively were collected. The samples were collected during the period 20th March, 2024 to last week of October, from different locations of farms and household-maintained sheep and goat in Andhra Pradesh like Bahubalendrunigudem, Gannavaram. Manikonda, Mustabad, Chekavaram, Nidamanuru, Chinna agiripalli and Sanvergudem. Sterile, cotton- tipped swabs were used to collect from the nasal nares and rectum, then placed into normal saline. Transportation was done on ice and immediately stored at 4°C for a minimum of 24 h until cultured/ enriched (Lyskova *et al*., 2007). The sample collection details are presented in Table 1.

Molecular confirmation of *K. pneumoniae* by PCR

Extraction of DNA by boiling and snap chilling method

An overnight culture of *K. pneumoniae* was centrifuged and resulting pellet was resuspended in 400 µL of nuclease-free water and later heated in a water bath for 10 minutes. The tube was promptly placed on chilled ice for 20 minutes, followed by centrifugation at 8000 rpm for 5 minutes at 4°C. The supernatant was then used as the DNA template for various PCR experiments. DNA concentration was measured using a Nanodrop 200C spectrophotometer (Thermo Scientific, USA) and adjusted to 150 ng for further molecular analysis. Pure DNA samples, with an optical density ratio of 1.8 to 2.0 at 260/280 nm, were stored at -20°C until use.

Molecular confirmation of *K. pneumoniae* isolates was done by PCR using oligonucleotide primers targeting species-specific genes (Table 2). The details of the standardized thermal cycling conditions are mentioned in Table 3.

Molecular detection of ESBL genes in *K. pneumonia* isolates

All the confirmed *K. pneumoniae* isolates were subjected to multiplex PCR for detection of ESBL genes, bla_{TEM}, $bla_{CTX\text{-}M,}$ bla_{SHV} and bla_{OXA} (Table 4). The m-PCR assay was conducted with an optimized PCR mix of 25µL, under standardized thermal cycling conditions (Table 5).

Table 1 Details of sample collected

PLACE	Type of sample				TOTAL
	Sheep		goat		
	Nasal (SN)	Enteric (SE)	Nasal (GN)	Enteric (GE)	
Bahubalendruni gudem	-	5	3	-	8
Gannavaram	6	7	8	6	27
Manikonda	5	3	3	2	13
Mustabad	2	1	7	5	15
Chekavaram	6	-	1	2	9
Nidamanuru	4	4	1	4	13
Chinna agiripalli	3	2	1	4	10
Sanvergudem	-	3	1	5	9
	26	25	25	28	104

Table 2. Species-specific oligonucleotide primers for *K. pneumoniae*

	Target gene	**Nucleotide sequence (5'-3')**	**Amplicon size(bp)**
K. pneumoniae	*rpoB*	CAACGGTGTGGTTACTGACG TCTACGAAGTGGCCGTTTTC	108

Table 3: Standardized thermal cycling conditions for *K. pneumoniae*

	Standardized conditions		
Steps	**Temperature**	**Duration**	**No. of cycles**
Initial denaturation	95℃	5 min	1
Denaturation	95℃	1min	
Annealing	55℃	1min	35
Extension	72℃	2 min	
Final extension	72℃	10 min	1

Table 4: Oligonucleotide primers used for theodetection of ESBL genes in K.pneumoniae (Bobbadi et al., 2019)

S.No	**Target**	**Nucleotide sequence**	**Amplicon size (bp)**
1	bla_{TEM}	CATTTCCGTGTCGCCCTTATT C	800
		CGTTCATCCATAGTTGCCTGAC	
2	bla_{SHV}	AGCCGCTTGAGCAAATTAAAC	713
		ATCCCGCAGATAAAATCACCA C	
3	bla_{OXA}	GGCACCAGATTCAACTTTCAAG	564
		GACCCCAAGTTTCCTGTAAGTG	
4	$b1a_{CTX-M}$	TTAGGAATGTGCCGCTGTA	688
		CGATATCGTTGGTGGTACCAT	

Table 5: Standardized thermal cycling conditions for detection of ESBL genes in K.pneumoniae by m-PCR (Bobbadi et al., 2019)

Steps	Standard conditions	Cycles
Initial denaturation	95ºC for 5 min	1
Denaturation	94ºC for 40 sec	
Annealing	60ºC for 40 sec	30
Extension	72ºC for 1min	
Final extension	72ºC for 7 min	1
Hold/stand by	4ºC for 10 min	---

Table 6: Oligonucleotide primers for *K. pneumoniae* virulence genes (Kot *et al*., 2023)

S N	Target gene	Nucleotide sequence	Amplicon size (bp)
1	*fimH*	TGCTGCTGGGCTGGTCGATG GGGAGGGTGACGGTGACATC	688

Table 7: Standardized thermal cycling conditions for detection of virulence genes in *K. pneumoniae* (Kot *et al.,* 2023)

Steps	Standard conditions	Cycles
Initial denaturation	95°Cfor 4 min	1
Denaturation	95°Cfor 0.5 min	
Annealing	56°C for 0.5 min	35
Extension	72°Cfor 1min	
Hold/stand by	4°Cfor ∞	---

Table 8: Molecular confirmation of *K. pneumoniae* along with the genetic determinants of its antibiotic resistance and virulence genes

Sno	Sample Id	Species specific 16s rRNA	Virulence gene	Esbl
1	GN3	+	+	TEM
2	SN2	+	+	
3	SE6	+	+	TEM
4	SE7	+	+	
5	GN4	+	+	TEM
6	GN7	+	-	
7	GN10	+	-	
8	GE1	+	+	TEM
9	GE3	+	-	
10	SN8	+	+	
11	SN10	+	-	
12	SE13	+	+	
13	SE15	+	+	
14	GN14	+	-	
15	GE7	+	+	
16	SN12	+	+	TEM
17	SE16	+	+	
18	GN16	+	+	
19	GN19	+	+	TEM
20	GE9	+	+	
21	GE11	+	+	
22	SN15	+	+	TEM
23	SN17	+	+	
24	SN18	+	+	
25	GN22	+	+	
26	GE15	+	+	TEM
27	SN22	+	+	
28	SE18	+	+	
29	SE20	+	+	
30	GE17	+	+	TEM
31	SN24	+	+	
32	SN25	+	+	
33	SE22	+	-	TEM
34	GE20	+	-	
35	GE23	+	+	TEM
36	SE24	+	+	
37	SE25	+	+	
38	GN25	+	-	TEM
39	SN5	+	+	
40	GE26	+	+	
41	GE27	+	+	
42	GE28	+	+	TEM

Electrophoresis of PCR amplicons on a 1.5% agarose gel stained with 2.5µg of ethidium bromide/mL in Tris-Borate EDTA (TBE) buffer was performed. The PCR products were visualized in the Gel documentation system after being electrophoresed at 70 V for 60 minutes in submerged gel electrophoresis equipment (BIORAD, UK). The sizes of the PCR products were confirmed using a quantitative DNA ladder (SRL, Mumbai). For PCR

assays, distilled water was utilized as a negative control. Characterized positive samples for the targeted genes is used as positive control.

Molecular detection of Virulence genes in *K. pneumonia* isolates

All the confirmed *K. pneumoniae* isolates were subjected to PCR for *fimH* gene (Table 6). PCR assay was conducted with an optimized PCR mix of 25 µL, under standardized thermal cycling conditions (Kot *et al*., 2023) (Table 7).

Results

Molecular confirmation of the *Klebsiella pneumonia* isolates

All the 42 isolates (22 enteritis and 20 pneumonia) that were culturally confirmed as *K. pneumoniae* were subjected to species specific confirmation by PCR using 16S rRNA out of which 42 isolates yielded a specific PCR product at 108 bp (Figure No. 1).

Detection of genetic determinants of ESBL in *K. pneumoniae* by m-PCR

All the 42 positive isolates that were confirmed as *K. pneumoniae* by PCR test were tested for the detection of ESBL genes using m-PCR. PCR products of 800 bp size were yielded for bla_{TEM} gene (Figure No.8). Out of the 42 isolates, 13 (30.9%) isolates were positive for the bla_{TEM} ESBL genes. The ESBL genes selected in the present study were not detected in 29 isolates (69.0 %) (Table 8).

Detection of genetic determinants of virulence for *K. pneumoniae* by PCR

All 42 isolates were assessed for presence of virulence genes (*fimH).* Out of the 42 isolates, *fimH* gene with amplicon size of 688 bp was detected in 34 (80.9%) isolates (Figure No: 3, Table 8).

Discussion

Klebsiella pneumoniae is an opportunistic pathogen, and the emergence of multidrug-resistant (MDR) strains in this bacterium has been linked to infections such as pneumonia and enteritis in small ruminants. In the present study, 42 (40.3%) isolates yielded a specific PCR product of 108 bp and similar findings were also reported previously (Bobbadi *et al*., 2021; Hassan *et al*., 2011; Mahrous *et al*., 2023; Singh *et al*., 2024).

All the 42 positive isolates that were confirmed as *K. pneumoniae* by PCR test were tested for the detection of ESBL genes using m-PCR. Out of the 42 isolates, 13 (30.9%) isolates were positive for the presence of bla_{TEM} gene with product size of 800 bp. The ESBL genes selected in the present study were not detected in 29(69%) isolates. Charrouf *et al*. (2014) examined antibiotic resistance in 68 ESBL producing *Klebsiella pneumoniae* strains isolated in Lebanon. Their findings showed that 88.2% of the isolates carried the *SHV* gene, and 86.7% carried the *CTX-M* gene. Additionally, 4 out of 5 of the isolates were found to harbor the *OXA-48* gene, which is associated with carbapenem resistance.

Marques *et al*. (2019) characterized the population structure, antimicrobial resistance, and virulence genes of 104 *Klebsiella* spp. isolates obtained from companion animals and human with urinary tract infections (UTIs) from University of Lisbon. The $bla_{CTX\text{-}M\text{-}15}$ gene was detected in over 80% of the third-generation cephalosporin (3GC)-resistant strains. The high-risk clonal lineage *K. pneumoniae* ST15 was found to be predominant among the companion animal isolates. Yang *et al*. (2019) studied *Klebsiella pneumoniae* isolates from human and bovine mastitis samples in New York. They reported the presence of a unique IncN-type plasmid, pC5, which coharbored the $bla_{CTX\text{-}M\text{-}1}$ and *mph(A)* genes, conferring resistance to cephalosporins and macrolides respectively. Banerjee *et al*. (2020) reported the presence of $bla_{CTX\text{-}M\text{-}15.2}$, $bla_{CTX\text{-}M\text{-}225}$, and $bla_{CTX\text{-}M\text{-}197}$ genes in *Klebsiella* isolates obtained from companion animals in Kolkata. A high prevalence of the $bla_{CTX\text{-}M\text{-}15}$ gene was observed in the isolates, and all *AmpC* beta-lactamase (ACBL)-producing isolates carried the *blaAmpC* gene.

Virulence factors associated with *K. pneumoniae*

The pathogenicity of *K. pneumoniae* is largely attributed to its diverse virulence genes, which facilitate adhesion to host tissues, evasion of immune defenses, and persistent infections (Riwu *et al*., 2022). These virulence factors include genes linked to capsule synthesis, siderophore production, fimbrial adhesins, and toxins, all of which contribute to its adaptability and infection capability. Understanding the distribution and function of these genes in

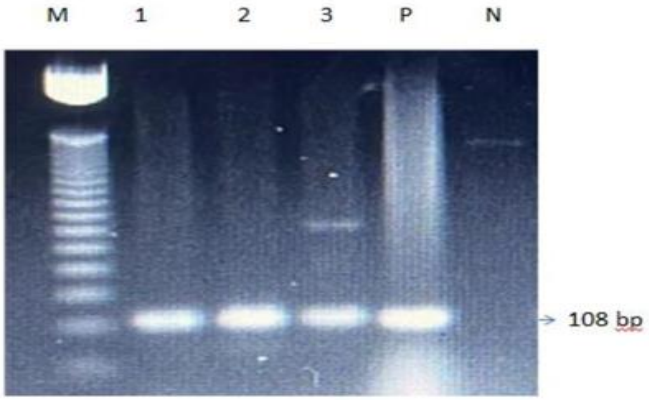

Figure 1: PCR for detection of *K. pneumoniae*

Lane M: DNA marker (50bp), Lane 1: SE7, Lane 2: SN2, Lane 3: GE3, Lane P: Positive control, Lane N: Negative control

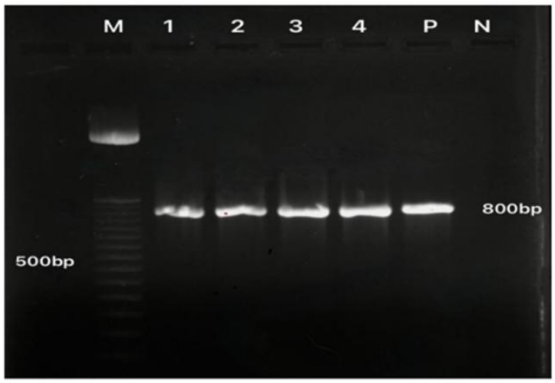

Figure No. 2: Multiplex PCR for detection of ESBL genes of *K. pneumoniae*

Lane M: DNA marker (50bp), Lane 1: SE7, Lane 2: SN2, Lane 3: GE3, Lane P: Positive control, Lane N: Negative control

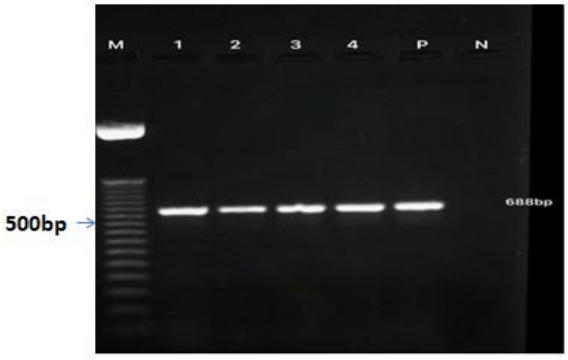

Figure No. 3: Polymerase Chain Reaction for detection of *fimH* virulence gene of *K. pneumoniae*

Lane M: DNA marker (50bp), Lane 1: SE7, Lane 2: SN2, Lane 3: GE3, Lane P: Positive control, Lane N: Negative control

small ruminant infections is crucial for devising effective management and treatment strategies. All 42 isolates were assessed for presence of virulence genes (*fimH).* Out of the 42 isolates, *fimH* gene with an amplicon size of 688 bp was detected in 34 (80.9%) isolates and this study was in agreement with the observations reported by Candan *et al*., (2015), Remya *et al*., (2020) and Anis *et al*. (2021) 84%, 89% and 86.7% isolates were positive for *fimH* gene respectively. Observations of the present study were slightly less than the results reported (91.5%) by Yang *et al*. (2019).

Remya *et al*. (2020) studied 370 human samples in Chennai, *K.pneumoniae* was isolated from 345 samples all of them carried multiple virulence genes in them 90% of the isolates carried *entB* gene followed by *FimH*(89.1%), *uge*(48.6%), *YbtS*(44.3%), *Kfu*(27.8%), aerobactin (5.4%), *rmpA*(5.1%), K2A(2%), *allS*(1%) and *magA*(0.2%).

Conclusion

The study reported a moderate prevalence of ESBL genes of only TEM gene and higher prevalence of virulence gene *fimH*, which shows significant therapeutic and infection control challenges in the area studied.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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