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Clinical *Klebsiella pneumoniae* isolates and their efflux pump mechanism for antibiotic resistance challenge

Ali A. Kadhum* , Mohammed H. Khudor  and Hanaa K. Ibraheim 

Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq

ABSTRACT

Background: *Klebsiella pneumoniae* is a serious pathogen that causes many disorders in humans and animals. *Klebsiella pneumoniae*, which is one of the most important pathogens in hospitals, often causes many clinical manifestations, including pneumonia, urinary tract infections, and meningitis. Interest in this bacterium has increased due to the increasing incidence of infection caused by it, as well as its high resistance to antibiotics, especially broad-spectrum antibiotics.

Aim: This study showed the efflux pump mechanism of clinical *K. pneumoniae* isolates and antibiotic resistance in samples collected from sheep and human respiratory tract infection in southern Iraq.

Methods: Three hundred samples were collected, and the samples included: 150 nasal swabs from sheep and 150 sputum samples from humans. Through bacteriological and biochemical examinations. The isolates were identified *K. pneumoniae* isolates were also confirmed by 16S rRNA. Susceptibility testing of the antibiotics used in the study. To determine the phenotypic efflux pump activity, the agar ethidium bromide cartwheel method was used.

Results: Of 150 sputum human specimens and 150 nasal swabs from sheep were tested, 25 and 17 *K. pneumoniae* species isolates from patients and sheep, respectively, for the resistance of the bacteria isolated from humans to antibiotics. The highest rate of resistance was to piperacillin (88%), and the lowest rate was to antibiotics (36%), imipenem. The highest of bacterial susceptibility to the antibiotic imipenem was (44%) and (36%) for levofloxacin, respectively. For the bacterial isolates from sheep, the highest percentage of resistance to rifampin was (82.3%), and the highest percentage of sensitivity was to imipenem and Levofloxacin antibiotics. The results showed that most of the 39 bacterial isolates (92.8%) possessed an efflux pump mechanism. The result of genotyping to identify the efflux pump genes *tolC* and *acrAB* revealed that all isolates carried the genes.

Conclusion: All the isolates were resistant to antibiotics, and the bacterial isolates under study most possess the efflux pump mechanism. All bacteria also have efflux pump genes, and this gives the bacteria more resistance against many antibiotics.

Keywords: *Klebsiella pneumoniae*, Efflux pump, Antibiotic resistance.

Introduction

Klebsiella pneumoniae is the most important cause of nosocomial infections belonging to the Enterobacteriaceae Gram-negative family (Miftode *et al.*, 2021; AlKhafaji *et al.*, 2024). Bacteria are the cause of some serious diseases when the immune defenses are weak, especially acute pneumonia. *K. pneumoniae* is considered an opportunistic pathogen that causes pneumonia (Carvalho *et al.*, 2021; Al-Kanany *et al.*, 2023).

Some animal products, particularly animal proteins and milk, have become important for global population growth. Therefore, it is necessary to prevent bacterial infections in animals and ruminants, and this is what veterinarians have sought to do (Al-Tememe and Abbas, 2022; Abdulla *et al.*, 2024). There are many

virulence factors shared between *K. pneumoniae* and other bacteria, which enable the transmission of *K. pneumoniae* bacteria, especially in hospitals. The most important of these factors is resistance to antibiotics of more than 80%. Immunocompromised patients can also be infected with enterobacteriaceae, most notably *K. pneumoniae* bacteria (Thamer and Shareef, 2022; Baqer *et al.*, 2023).

Klebsiella pneumoniae has developed multiple mechanisms to resist antibiotics. The efflux pump mechanism is a system based on a specific protein that is important in expelling antibiotics and some toxic substances out of the cell, where its work is with membrane permeability to reduce the concentrations of these substances (Effah *et al.*, 2020; Ashwath and Sannejal, 2022). The RND family is one of the most

*Corresponding Author: Ali A. Kadhum. Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq. Email: ali.abd@stu.edu.iq

important pump families according to its classification, as it includes Gram-negative bacteria, including *K. pneumoniae*, which contains the *acrAB-tolC* gene and the diffusion protein in the compartment, as well as a protein to the inside of the channel in the membrane, which releases materials into the environment outside the cell (Reza et al., 2019). Because efflux pumps have the ability to eliminate multiple medicines, including erythromycin, B-lactams, fluoroquinolones, and chloramphenicol, they are the most significant mechanism of antibiotic resistance (Li et al., 2021).

However, it is well known that certain bacterial efflux pumps export host-derived antimicrobial compounds in addition to antibiotics and other materials like dyes and detergents (Islamieh et al., 2018; Swedan and Aldakhily, 2024). Many substances, notably antibiotics, are pumped out of bacterial cells via an ATP-dependent transport system (Reza et al., 2019). Antibiotic resistance is related to resistance to efflux pumps, and this has been studied (Kahlmeter et al., 2019). Many gram-negative bacteria also contribute to environmental pollution and thus the occurrence of diseases in society through their ease of transmission among people (Al-Shanawa, 2013).

Klebsiella has mechanisms that cause multiple diseases in humans and animals, as it expresses on its surface a soft lipopolysaccharide (LPS with O antigen) and a capsular polysaccharide (K antigen) (Kadhum and Khudor, 2021). In this study, we isolated *Klebsiella* bacteria from samples of human and sheep with respiratory infection. The isolates were then identified using advanced bacteriological methods, given the critical importance of investigating pathogens that pose public health risks (Mazaal et al., 2021). This study aimed to show the efflux pump mechanism of clinical *K. pneumoniae* isolates and antibiotic resistance in samples collected from sheep and human respiratory tract infections in southern Iraq.

Materials and Methods

Specimens collection and bacterial identification

Three hundred specimens were collected from the AL-Nasiriya Teaching Hospital and Veterinary Hospital, and the specimens included 150 nasal swabs from sheep. Nasal swabs taken from sheep are a rapid, nonsurgical method that provides accuracy and effectiveness in diagnosing respiratory diseases, and 150 human sputum samples. From the patient records, information about each patient was collected. The samples were then taken and transferred to the laboratory in special containers for the purpose of culturing them on MacConkey agar and incubating them at 37°C for 24 hours. Bacterial cells were stained by Gram staining, and isolates were identified by p Vitek-2 techniques. Biochemical tests were employed to confirm *K. pneumoniae* as per the methods described by (Jamalludeen, 2020).

Antibiotic susceptibility testing

The diffusion method was carried out according to the guidelines of the European Committee for Susceptibility Testing. The antibiotic discs were selected according to the scientific standards. Ten antibiotics were selected against the bacterial isolates after planting them on Mueller-Hinton agar medium. Then, the antibiotics were placed. After that, the plates containing the bacteria and antibiotics were incubated at 37°C for 24 hours. The results were compared with the latest standard tables in the Clinical Laboratory Standards Institute (CLSI) for the year 2023 (Ibraheim et al., 2023).

Mechanism efflux pump

The agar ethidium bromide cartwheel method (Gharbi et al., 2024). Ethidium bromide at concentrations of 0.5, 1, 1.5, and 2 mg/l was incorporated into Müller Hinton agar to evaluate efflux pump phenotypes in *K. pneumoniae*. Bacterial strains were streaked in a linear cartwheel formation adjacent to a negative control, followed by overnight incubation at 37°C. Efflux-mediated fluorescence was detected using a Uvitec Cambridge UV transilluminator (Sepehr et al., 2022).

Molecular assay

By (Bromega/United States), we extracted genomic DNA from *K. pneumoniae* isolates according to the instructions and methodology of Gram-negative bacteria. Conventional PCR amplification of genes using universal primers (Table 1). The PCR program was performed as described in Table 2.

Statistical analysis

The results of the present experiment were statistically analyzed using the Chi-square test, and the variances at $p < 0.05$ are significant statistically.

Ethical approval

The Laboratory Animal Ethics Committee granted the license and approval to conduct the research at the College of Veterinary Medicine, University of Basra.

Results

Sample collection and identification of bacteria

Of 150 sputum human specimens and 150 nasal swabs from sheep were tested, 25 and 17 *K. pneumoniae* species isolates from patients and sheep, respectively, with respiratory infections were identified using cultural methods. Biochemical tests of the bacterial isolates gave negative results for the indole, methyl red, and oxidase tests, while the bacterial isolates produced catalase and urease enzymes and gave positive results for the Voges-Proskauer and citrate consumption tests. Suspected isolates were also confirmed using the Vitek2 system, which was supported by *K. pneumoniae* iron through phenotypic methods on culture media. The results of identifying all isolates were positive (100%). Molecular methods through PCR amplification of *16S rRNA* using universal primers for 16S rRNA of *K. pneumonia* (Fig. 1).

Table 1. Sequences and sizes of primers used to amplify the *acrAB* and *tolC* genes.

Gene	Sequence primer (5-3)	Size (bp)	Reference
<i>16sRNA</i>	F: AGAGTTTGATCCTGGCTCAG R: TACGGTTACCTTGTACGACTT	1,500	Monawer (2025)
<i>AcrAB</i>	F: ATCAGCGGCCGATTGGTAAA R: CGGGTTCGGGAAAATAGCGCG	312	Ahmed and Mawlood (2023)
<i>tolC</i>	F: ATCAGCAACCCCGATCTGCGT R: CCGGTGACTTGACGCAGTCCT	527	

Table 2. Special program for *acrAB*, *tolC*, and *16sRNA* gene amplification.

Stage	<i>acrAB</i> gene amplification			<i>tolC</i> gene amplification		
	Temperature\°C.	Time	No. of cycles	Temperature\°C.	Time	No. of cycles
Initial denaturation	94	5 minutes	1 cycle	94	5 minutes	1 cycle
Denaturation	94	45 seconds		94	45 seconds	
Annealing	53	35 seconds	40 cycle	57	45 seconds	35 cycle
Extension	72	45 seconds		72	45 seconds	
Final extension	72	7 minutes	1 cycle	72	7 minutes	1 cycle

Antibiotic susceptibility profile for *K. Pneumoniae*

The resistance and sensitivity of bacteria to the selected group of antibiotics under study were determined by the Kirby-Bauer method. Then, the inhibition zone of *K. pneumoniae* bacteria was shown, where the area was measured in millimeters, compared to the standard zone of inhibition as stated in CLSI (2023).

The findings revealed that. The majority of sensitive isolates from humans and sheep were for IMipenem. While there were 22% resistant isolates for Piperacillin antibiotics in humans, there were 14 resistant isolates (sheep) for rifampin, as well as some isolates were intermediate or sensitive (Tables 3 and 4). The percentage of resistance to antibiotics was revealed as follows in humans: the highest percentage of resistance to Piperacillin was (88%) and to ceftriaxone was (76 %) whereas the lowest percentage was (9\36%) to the antibiotic IMipenem and (10\40%) to ciprofloxacin whereas the highest percentage of bacterial susceptibility to the antibiotic IMipenem was (11\44%) and (9\36%) to Levofloxacin (Table 3).

Regarding the bacterial isolates from sheep, the highest rate of resistance to Rifampin was (14/82.3%), as well as the highest rate of susceptibility to the antibodies IMipenem and Levofloxacin. The results revealed that the antibiotic susceptibility of *K. pneumoniae* isolated from human and sheep. The results showed a significant ($p < 0.05$) increase in resistance for antibiotics, while the results were obtained significant ($p < 0.05$) decreased in sensitive for antibiotics but the results non-significant ($p > 0.05$) difference in intermediate for antibiotics (Tables 3 and 4).

Mechanism efflux pump

The bacterial isolates were tested to determine the phenotypic appearance of the efflux pump using the Cartwheel test, and the results showed that most of the bacteria, 39 isolates, 92.8% possess the efflux pump mechanism to resist antibiotics. The isolates containing the efflux pump exhibited fluorescent emission under a UV transilluminator (Fig. 2). The result of genotyping to determine the *tolC* and *acrAB* efflux pump genes showed that all isolates carried the genes. (Figs. 3 and 4).

Discussion

Klebsiella pneumoniae has the ability to cause serious diseases in humans and animals because of its many virulence characteristics; thus, an immune response occurs as an immune reaction against the infection. As observed during our study, the incidence of *K. pneumoniae* in sheep was lower than in human (Karampatakis *et al.*, 2023; Mahrous *et al.*, 2023).

The bacteria were grown on MaConkey agar, after which biochemical tests were performed. Then, the identity of the suspected *K. pneumoniae* was determined by the VITEK2 system, where the results were all *K. pneumoniae*, and this result was 100% consistent with previous studies, such as Su *et al.* (2023) and Al-Mousawi and Al-Daraghi (2022).

Because some isolates are more resistant to piperacillin and ceftazidime, which indicates an increase in the expression of the beta-lactamase enzyme, most of the bacterial isolates were sensitive to imipenem, and this is what was agreed upon with Mohammed *et al.* (2023) and Hou *et al.* (2024). It was shown that *K. pneumoniae* isolates are highly susceptible to imipenem, followed



Fig. 1. Gel electrophoresis, for amplification of *16S rRNA* 1500bp. of *K. pneumoniae* using conventional PCR, stained with RedSafe™ and visualized on a UV transilluminator. Marker (L): DNA ladder (100–3,000 bp), Lanes: (1–25) human sputum samples, (1–17) sheep nasal swabs samples.

Table 3. Antibiotic susceptibility of *K. pneumoniae* isolates from human patients.

Antibiotics	Resistance NO. (%)	Intermediate NO. (%)	Sensitive NO. (%)
Rifampin	17 (68)	5 (20)	3 (12)
Ceftazidime	18 (72)	3 (12)	4 (16)
Cefepime	16 (64)	4 (16)	5 (20)
Piperacillin	22 (88)	1 (4)	2 (8)
Ceftriaxone	19 (76)	4 (16)	2 (8)
Tetracycline	17 (68)	5 (20)	3 (12)
Azithromycin	13 (52)	5 (20)	7 (28)
Levofloxacin	11 (44)	5 (20)	9 (36)
Ciprofloxacin	10 (40)	8 (32)	7 (28)
IMipenem	9 (36)	5 (20)	11 (44)
$p < 0.05$	0.002	0.068	0.001

by ceftazidime and ciprofloxacin. This result was in agreement with the study by Jasim *et al.* (2021) reported that bacteria are sensitive to ciprofloxacin. However, *K. pneumoniae* was resistant to tetracycline and ceftriaxone. *Klebsiella pneumoniae* bacteria isolated from sheep showed higher resistance to most antibiotics compared to human isolates, as they showed more resistance to rifampin (82.3%), ceftriaxone, and tetracycline (64.7%) Among these isolates, varying degrees of sensitivity were observed, as they were more sensitive to imipenem and levofloxacin, respectively

(47%) and 35.2% (Oraibi *et al.*, 2020; Yadav, 2020). The presence of high resistance in bacterial isolates of sheep may be due to the irresponsible use of antibiotics in livestock feed, which may lead to resistance that affects the health of animals and humans, as well as the direct exposure of animals to environmental factors such as environmental pollution resulting from the discharge of antibiotics into water and fertilizers that are not subject to health control, which gives bacteria greater resistance and thus increases bacterial resistance (Larsson and Flach, 2022).

Table 4. Antibiotic susceptibility of *K. pneumoniae* isolated from sheep.

Antibiotics	Resistance NO. (%)	Intermediate NO. (%)	Sensitive NO. (%)
Rifampin	14 (82.3)	2 (11.7)	1 (5.8)
Ceftazidime	10 (58.8)	4 (23.5)	3 (17.6)
Cefepime	9 (52.9)	5 (29.4)	3 (17.6)
Piperacillin	13 (76.4)	2 (11.7)	2 (11.7)
Ceftriaxone	11 (64.7)	4 (23.5)	2 (11.7)
Tetracycline	11 (64.7)	5 (29.4)	1 (5.8)
Azithromycin	10 (58.8)	3 (17.6)	4 (23.5)
Levofloxacin	6 (35.2)	5 (29.4)	6 (35.2)
Ciprofloxacin	7 (41.1)	7 (41.1)	3 (17.6)
IMipenem	4 (23.5)	5 (29.4)	8 (47)
$p < 0.05$	0.002	0.061	0.000

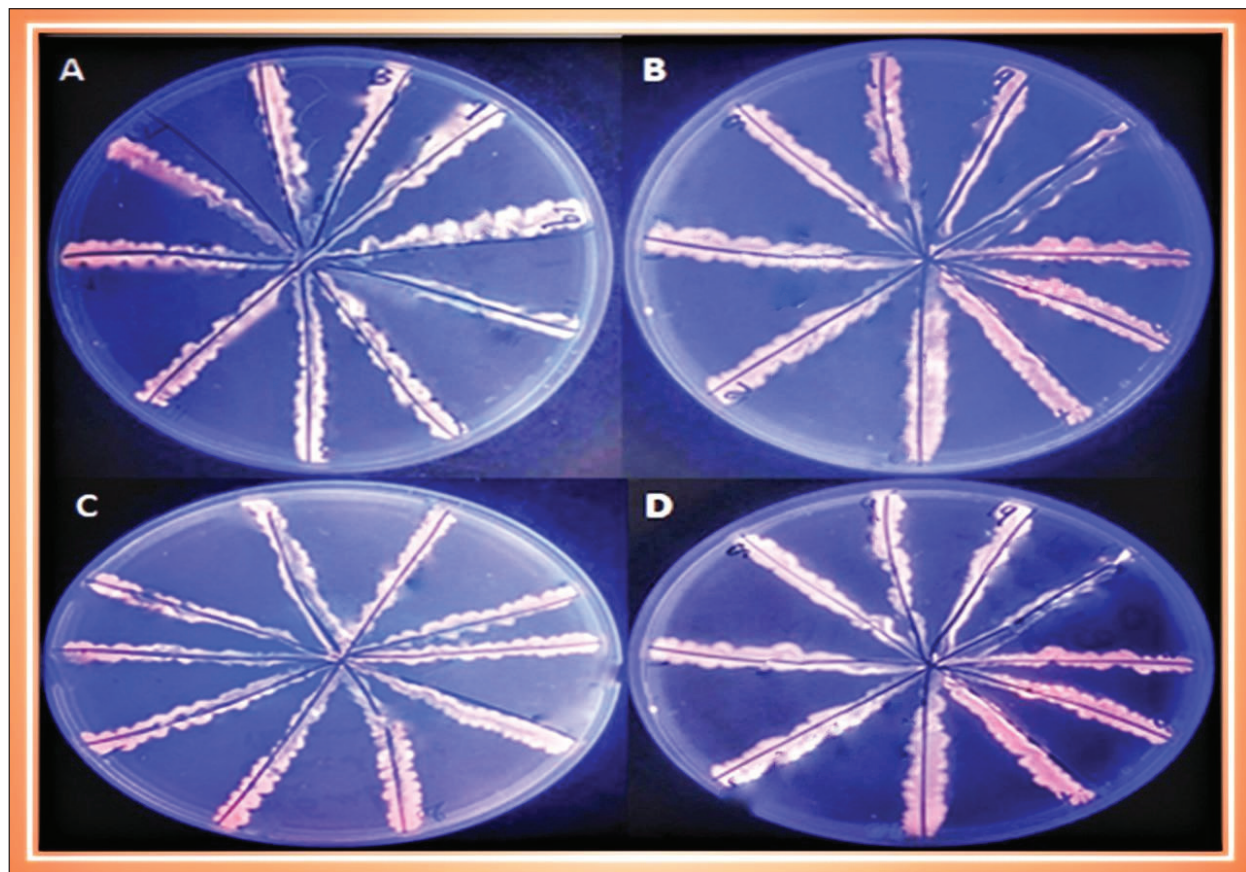


Fig. 2. Efflux pump phenotype using the Cartwheel test for *K. pneumoniae* in different EtBr concentrations. A: 0.5 mg/l B: 1 mg/l C: 1.5 mg/l D: 2 mg/l. The isolates containing the efflux pump exhibited fluorescent emission under a UV transilluminator.

All of the *K. pneumoniae* isolates investigated in this study possess an active efflux pump. This could explain that the active flow pump plays a role in the level of antibiotic resistance (Szabo *et al.*, 2018; Maurya *et al.*, 2019), where the role of the efflux

pump in quinolones was explained, resistance to β -lactam antibiotics in *K. pneumoniae*. During the current study, we found that all isolates possessed an efflux pump system, which is consistent with the study of Akinpelu *et al.* (2020). In this study,

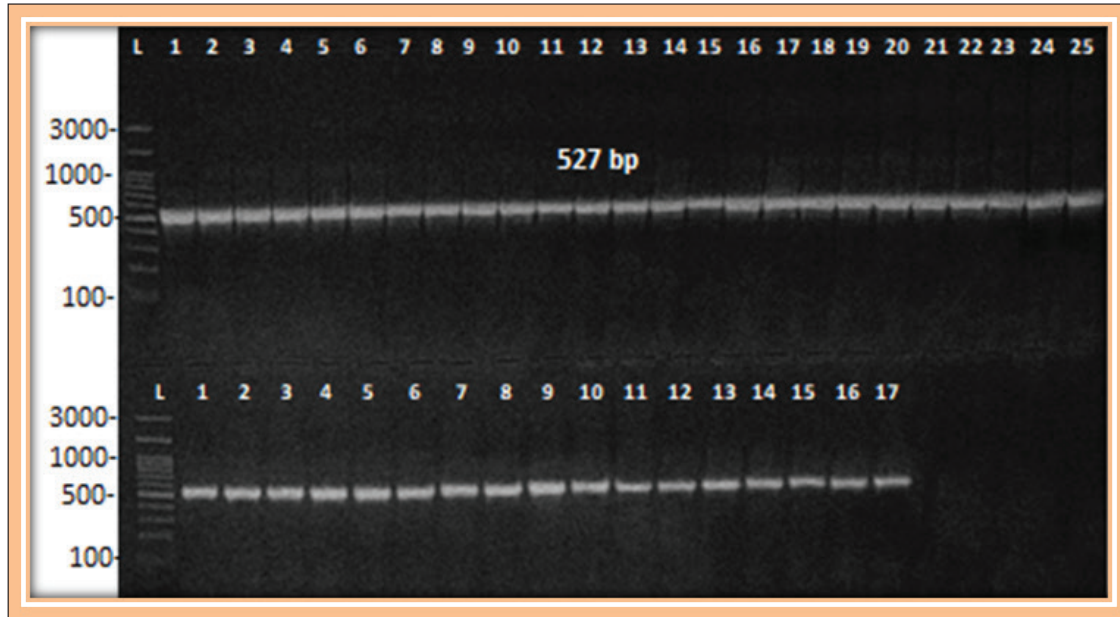


Fig. 3. Conventional PCR results. Gel electrophoresis for *tolC* gene size 527 bp with RedSafe™ and visualized on a UV transilluminator. Lane (L): 300 bp. ladder, lane (1–25) Positive result for human isolates, lane (1–17) positive result for sheep isolates.

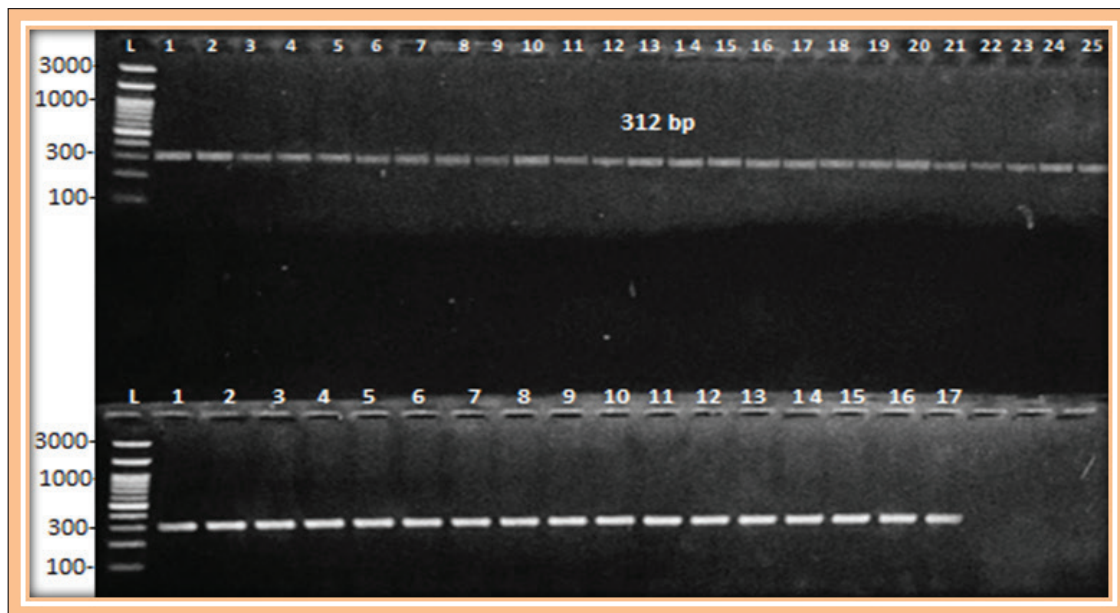


Fig. 4. Conventional PCR results. Gel electrophoresis for *acrAB* gene size 312 bp. with RedSafe™ and visualized on a UV transilluminator. Lane(L): 300 bp. ladder, lane (1–25) Positive result for human isolates, lane (1–17) positive result for sheep isolates.

the efflux pumps *acrAB* and *tolC* genes were identified. In both human and sheep isolates, it was 100%. These results are consistence to other results recorded by Suresh and Pillai (2024) and Ferreira (2019).

Conclusion

Most *K. pneumoniae* isolates were isolated from human patients with respiratory infections, as well as from sheep with respiratory infections. In addition, all the isolates were resistant to antibiotics, as it was

known that the isolates under study, most bacteria (39 isolates), 92.8% possess the efflux pump mechanism. All bacteria also have efflux pump genes, and this gives the bacteria more resistance against many antibiotics.

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Conflict of interest

The authors declare no conflict of interest.

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Author's contributions

All authors have conceived, designing, collecting data, analyzing and interpreting, composing, and preparing the first draft, writing, and revising the manuscript. All authors have reviewed and approved the published version of the work.

Data availability

All the information is contained in the paper.

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