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Molecular detection of virulence genes of *Klebsiella pneumonia* from Camels

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Abstract: Klebsiella pneumonia is recognized as one of the most important microorganisms of economic importance to the dairy industry worldwide, affecting almost all domestic animals. This study aimed to isolate K. pneumonia from Camels (60 nasal and fecal samples) appearing noticeable respiratory and GIT symptoms and clinical signs, antibiotics susceptibility investigation with frequency of some virulence factors genes. The results on differential media showed primary identification of 55% Klebsiella spp., 21% E. coli, 15% Enterococcus spp., and 9% Pseudomonas spp. However, the quantitative PCR for the 16S rRNA gene confirmed that 27 out of 33 suspected isolates were K. pneumonia. Multi-sequence alignment of this sequenced isolate showed a high identical score to K. pneumonia strains from Hong Kong and Nigeria. The results also showed that 100% of K. pneumonia isolates were resistant to vancomycin and highly sensitive to levofloxacin, trimethoprim, and ceftriaxone. The molecular detection showed some virulence factors genes (ESBL). Differences in gene comparison with recently reported research on bovine (90.0 bla- ctx, 100.0 int1, 60.0 int2, 60.0 kpc, 10.0 hemo, and 0.0% of bla-shv) were found. In conclusion, K. pneumonia isolated from camels has a high sensitivity towards many antibiotics with a lower rate of some virulence genes in comparison with those isolated from cows.
Keywords: K. pneumonia, Camel, Antibiotics susceptibility, ESBL, 16S rRNA.

1. Introduction

Camels represent one of the most spreading domestic animals in Iraq. Thus, studies of camel diseases are of considerable value in Iraq (Al-Yasari et al., 2023). Many pathogens can be harbored within the gastrointestinal tract of food-producing animals due to contamination of meat during processing, *K. pneumoniae* is an opportunistic bacterium commonly found in the gastrointestinal tract (Klaif et al., 2019; Al-Saadawe and Alsaadawi, 2022). *K. pneumoniae* is a member of Enterobacteriaceae which was observed as a significant pathogen responsible for acquired nosocomial and public infections (Shon, Bajwa, and Russo, 2013).

K. pneumoniae was connected with the formation of extended-spectrum β-lactamases (ESBL) that cause severe effects on human health due to Developing antimicrobial resistance in animal pathogens. Especially, penicillins and early cephalosporins like bla_{CTX-M} and bla_{SHV} genes (Enferad and Mahdavi, 2021). Integrons are conserved DNA sequences carried on episomal genetic structures that deliver a crucial approach for capturing and dispersion of the genes of antimicrobial resistance (ESBL) (Chen et al., 2013). Two conserved regions are involved within integrons that are neighboring variable regions holding single or multiple resistance genes (Fluit and Schmitz, 2004).

Detailed descriptions of their structure appeared as three genetic units constructed from integrase genes, gene cassettes (att), and a site of integration. Due to the integrons being immobile, the gene cassettes can be excised and integrated by the integrase to form the integron (Sepp et al., 2009). Despite their contribution to gene traffic, they also can be located within transposons or conjugative plasmids, as well as bacteria can acquire new genetic material (Mobarak-Qamsari et al., 2013). The most predominant genes are class 1 integrons, which are found in ESBL. Frequently, these are produced in medical bacteria of Enterobacteriaceae, such as *K. pneumoniae*. Less frequently, class 2 integrons could also be present in ESBL of *E. coli* and *K. pneumoniae*. In contrast, the production of class 3 integrons is very rare, although they are o found in ESBL (Pérez-Etayo, 2018). The cytolysis toxin (Hemolysin) is produced by some microorganisms that possess these virulence factors and act on erythrocytes for lysis. This is associated with the pathogenesis of these microorganisms (Curiao, 2010).

Hemolysins are significant causes of host damage due to facilitating bacteria dissemination. It also may cause alteration in host pathways, cell survival, cytoskeletal dynamics, and inflammatory response (Yigit, 2001). In 2001, the KPC-type enzyme was first described in North Carolina from *K. pneumoniae* strains that were resistant to carbapenem (Nordmann and Poirel, 2002). This enzyme was classified later as one of Ambler class A enzymes (NMCA, IMI, SME, GES, and KPC), which are acquired carbapenem-hydrolyzing β-lactamases (Ke et al., 2007). Although the description of this enzyme participation activates the process of spreading, the information on rapid spread and the responsible genetic elements needs to be studied further (Naas et al., 2008). There is an association between the KPC gene (carbapenemase) and the plasmid-borne transposon Tn*4401* that may cause rapid dissemination (Naas, 2008). This study aimed to detect *K. pneumoniae* in camels from Al-Muthanna province desert, Iraq, and examine the resistance profile to several antibiotics. In addition, we determined some of the virulence genes in these bacteria.

2. Methodology

Sixty nasal and fecal swabs were collected from camels suffering from respiratory infection (heavy nasal discharge and lacrimation), as well as signs of mild diarrhea. These camels were herded in the desert of Al-Muthanna province (30º12'N – 45º21'E), southern Iraq. This study was settled from November 2021 to February 2022. The samples were transported to the Microbiology laboratory in the veterinary college at the University of Al-Muthanna/ Iraq using sterile tools and transport media tubes for appropriate examination. Filled swabs of all samples were used for culturing on MacConkey agar, which is more specific

for gram-negative bacteria. The samples were prepared according to the manufacturer's instructions and incubated for 18 h at 37ºC. All isolates were then cultured on Chrome orientation media and incubated for 18 h at 37ºC. This is a differentiated media for some bacteria based on the morphological description of the colony and media color change (Lengfelder et al., 2019).

3. DNA Extraction

A colony of suspected *K. pneumoniae* isolates was inoculated for 16 h in nutrient broth. Genomic DNA extraction from 1 ml overnight growth samples was done using manufacturer instructions (Qiagen Kit, Hilden, Germany). The extracted DNA was examined by a Nanodrop spectrophotometer (Fisher Scientific, Loughborough, England) and then stored at -20 ºC in a freezer (Fisher Scientific, Loughborough, England) until use according to Ahmed, Al-Dabbagh, and Jwher (2021).

4. PCR detection of *K. pneumoniae*

Conventional PCR technique was carried out on all isolates for the detection of bacteria. All PCR samples were prepared as per manufacturer instructions (1.5 µl of 10 pMol forward and reverse primers, five µg genomic DNA, 12.5 Mastermix, and a complete volume of 25 µl of nuclease-free water. Primers for detecting *16S rRNA* were used to confirm isolation of *K. pneumoniae* (forward: 5' AGAGTTTGATCCTGGCTCAG 3', Reverse: 5' GGTTACCTTGTTACGACTT 3') (Heuer et al., 1997). PCR programs were set according to the annealing temperature of each primer: 95ºC for 5 minutes, 30 cycles at 94ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 45 seconds, and a final extension at 72ºC for 7 minutes, with a final hold at 4ºC (Thermo-Fisher GmbH, Schwerte, Germany). PCR products were then loaded on 1.5% agarose gel electrophoresis using 1X Tris-Borate- EDTA (TBE buffer), which was prepared by mixing Tris base 10.8 g (89 mM), boric acid 5.5 g (2 mM), 4 ml of 0.5M EDTA (pH 8.0), and then the components were dissolved to 1 L of distilled H2O and run for 60 min/80 volt. DNA bands were stained with ethidium bromide that was measured by a 3 Kbp DNA ladder to confirm the specific size of 16S rRNA genes (1500 bp) for the subjected bacteria. UV Transilluminator (Jlab Science, Ambala, India) for DNA detection was used for imaging.

Gene		Primer sequence	Tm	Amplicon	REF
bla-shv	F	ATGCGTTATATTCGCCTGTG	55	730 bp	(Mirnejad et al., 2013)
	R	TGCTTTGTTATTCGGCCAA			
	F	CGCTTTGCGATGTGCAG	55	550 bp	(Dillon et al., 2005)
	R	ACCGCGATATCGTTGGT			
Into1	F	CAGTGGACATAAGCCTGTTC	55	160 bp	Hossain et al. 2004
	R	CCCGAGGCATAGACTGTA			
Into2	F	CAGGGATATGCGACAAAAAGG	54	788 bp	Esmaeel & Sadeq, 2018
	R	GTAGCAAACGAGTGACGAAATG			
bla-kpc	F	GCTACACCTAGCTCCACCTTC	55	989 bp	(Molana et al., 2011)
	R	ACAGTGGTTGGTAATCCATGC			
hemo	F	CCGGAGCGTTTTTCGATTGG	57	413 bp	(Eftekhar et al. 2012)
	R	AGCATCCGGGTAAAAAGGGG			

Table 1 – Primers for PCR amplification of virulence factors genes of *Klebsiella pneumoniae.*

5. Partial sequencing of 16S rRNA gene

A purified sample (40 µl) of 16S rRNA PCR product was prepared and sent out to Macrogen Company (Seoul, South Korea) for sequencing. NCBI BLASTn engine was carried out to indicate the presence of remarkable homology with the expected target that covers a maximum portion of 16S rRNA gene within K. pneumoniae genome sequences; then the sequenced data was applied for multiple alignments using MEGA X software, Version MEGA 11.0.11 (Allenstown, USA) to construct a phylogenetic tree to discover the genetic variation between the subject strain with global strains of *K. pneumoniae*.

6. Antibiotic sensitivity test

Muller Hinton agar (HIMedia, Maharashtra, India) was prepared following manufacturer instructions for examining the antibiotic sensitivity of K. pneumoniae. Isolates were streaked on a plate by cotton swaps, then 10 different types of antibiotics discs (HIMedia, Maharashtra, India) like (amikacin 10µg, trimethoprim 10µg, amoxicillin 25µg, ceftriaxone 10µg, levofloxacin 5µg, vancomycin 30µg, tetracycline 10µg, nitrofurantion 100µg, cefixime 5µg, clarithromycin 5µg) were distributed on two plates, five different antibiotics on each plate. The incubation process was carried out for 16 h at 35º C, according to Ahmed (2021).

7. Detection of *K. pneumoniae* virulent genes

Based on the results of antibiotic sensitivity, *Klebsiella* isolates were used for the detection of virulent genes by conventional PCR to find the variation of these genes in comparison with the same bacteria in different animals. Primers of six different genes were designed for molecular detection (Table 1). PCR samples and settings were as mentioned above. All PCR products were also loaded on 1% agarose gel and run for 40 min/80 volts. A UV Transilluminator was used for DNA checking.

8. Results

8.1. Isolation and identification of *K. pneumoniae*

The results showed various morphology of colonies based on color and lactose fermentation. The colonies were pink mucoid and fermented, pink dry and fermented, brown non-fermented, and gray non-fermented. On the other hand, the growth on Orientation Chrome media after taking a single colony from MacConkey culture gave an initial indication of the type of bacteria according to morphological characteristics (Figure 1). The data showed *K. pneumonia*, *E. coli*, *Enterococcus*, and *Pseudomonas* spp. presence (Table 2).

Bacteria colony	MacConkey	Orientation Chrome agar	Number of Isolates	%
K. pneumonia	pink mucoid and lactose fermented	Metallic to dark blue color	33	55
E. coli	pink dry and lactose fermented	Dark rose to pink	13	21
Enterococcus	brown non lactose fermented	Turquoise to green	9	15
Pseudomonas	gray non lactose fermented	Creamy to transparent	5	9

Table 2 – Growth of bacteria colony on deferential mediums.

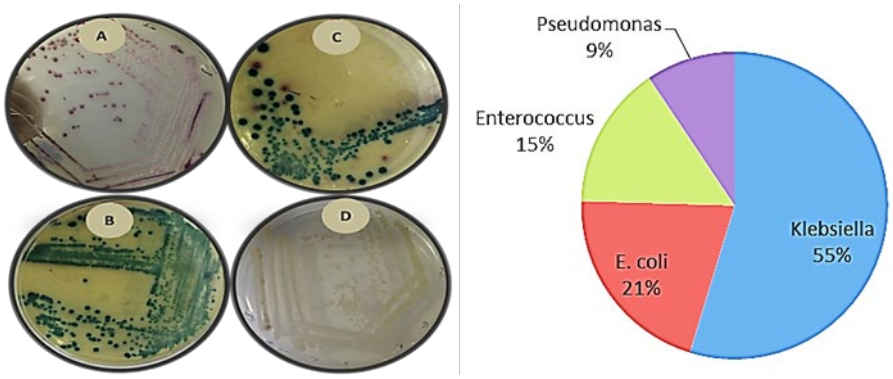

Figure 1 – Morphological examination of bacteria (left) and infection rate (right). The figure shows the growth of bacteria colonies on orientation Chrome agar. A- *E. Coli*, Pink colonies, B- *Enterococcus*, Green colonies, C- *Klebsiella*, Metallic Blue colonies, D- *Pseudomonas*, creamy color.

8.2. PCR detection of *K. pneumoniae*

According to the estimated results of Orientation Chrome agar, the suspected *K. pneumoniae* isolates were determined by PCR with expected 1500 bp product sizes. The agarose gel recorded 27 isolates (82%) of the total suspected isolates were *K. pneumoniae* (Figure 2).

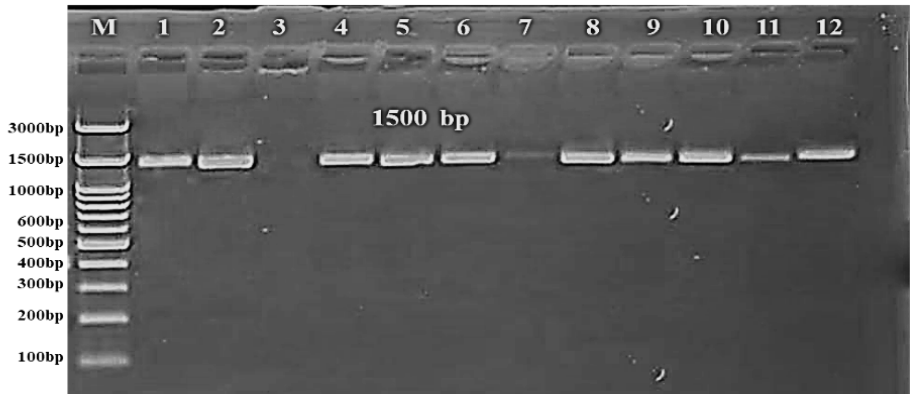

Figure 2 – PCR amplification of *16S rRNA gene.* Gel electrophoresis of PCR products of *16S rRNA* gene in suspected *K. pneumonia* isolates with particular molecular sizes (1500 bp). (M) 3 Kbp DNA marker. 1-12 represent isolates.

8.3. Phylogeny analysis

A sample of 16S rRNA PCR product was partially sequenced to confirm the presence of *K. pneumoniae* and then registered at the NCBI website under the name of *K. pneumoniae* strain NHVM1 (Accession number; OR364053.1). The results show a significant resemblance (99.8) of the nucleotide sequence of the subjected isolate with other isolates from Hong Kong, Nigeria, China, and India (Figure 3).

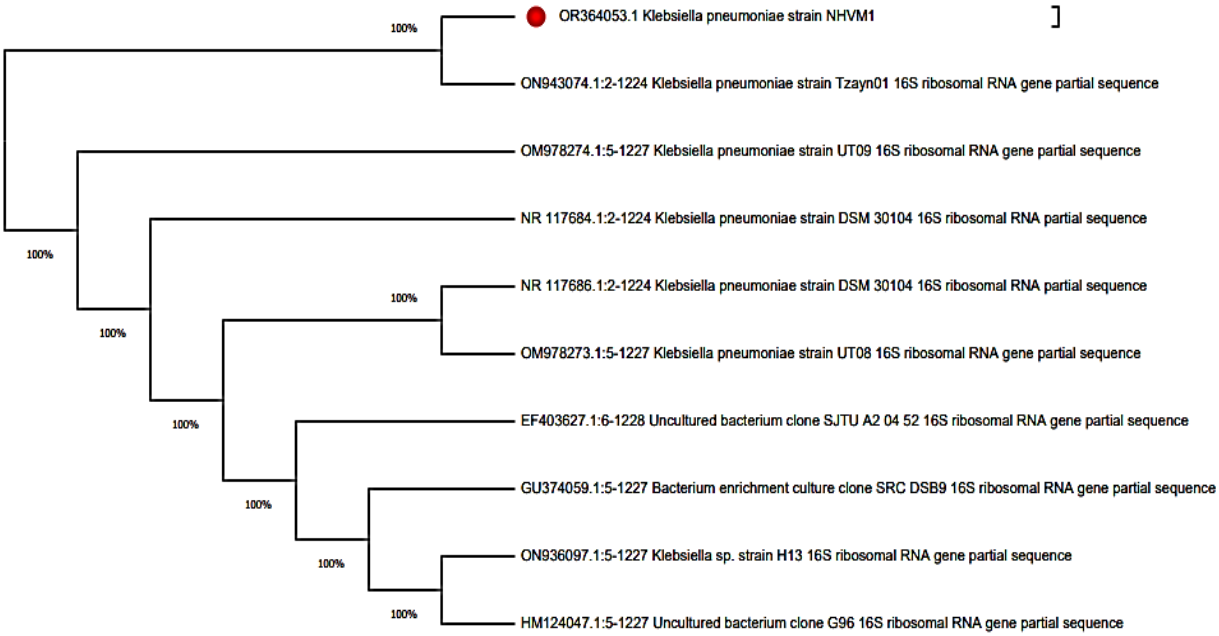

Figure 3 – phylogenetic tree analysis of *K. pneumoniae.* It shows the similarity approach between the new *K. pneumoniae* strain (NHVM1, red highlight) and other closely related global strains of *K. pneumoniae* according to the sequence of the 16S rRNA gene. The tree was constructed based on the neighbor-joining method. Numbers at nodes represent levels of bootstrap support (%) based on analysis of 1000 replications.

8.4. Antibiotic susceptibility test

The results showed that the *K. pneumoniae* isolates were highly sensitive to most of the subjected antibiotics based on standard measurements, especially levofloxacin, trimethoprim, and Ceftriaxone, which gave a large size of inhibition zone (over 30 mm) Figure 4. Meanwhile, the resistance was noticeable for vancomycin (100%) and amoxicillin (75%).

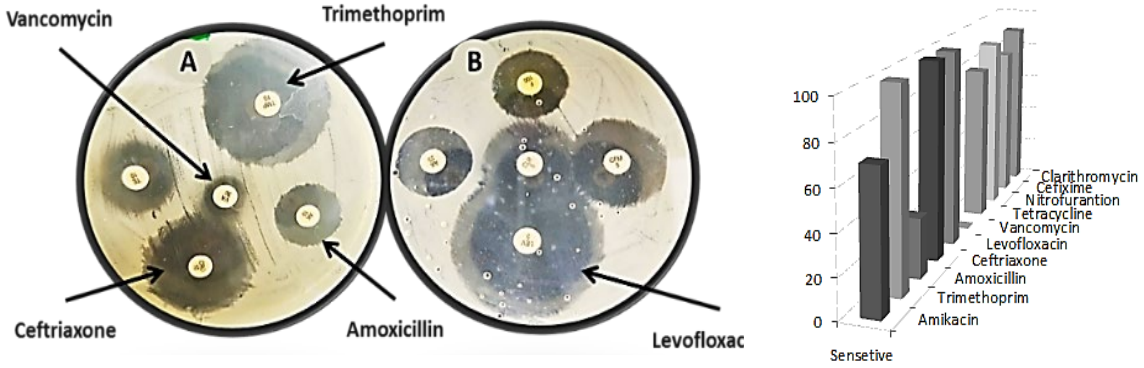

Figure 4 – Disc deffusion test and Antibiotics resistance patterns of *K. pneumoniae* isolates. It shows the resistance ratio of Klebsiella to Vancomycin and Amoxicillin. Although its sensitivity towards the rest of the applied antibiotics. The efficiency of Levofloxacin, Trimethoprim, and Ceftriaxone on this bacteria was very obvious as a zone of inhibition showed.

8.5. PCR detection of *K. pneumoniae* virulent genes

The results showed that the presence of *K. pneumoniae* virulent genes were variant. Intgron1 gene, which is responsible for capturing and dispersion of antimicrobial resistance genes, was detected in all *K. pneumoniae* isolates (100%). The extended-spectrum β-lactamase gene (*bla-ctx*) was found in (90%) of isolates. Genes of Intgron2 and carbapenem-hydrolyzing β-lactamase (*kpc*) were determined in (60%) of isolates. Additionally, erythrocytes lysis gene (Hemolysis) was detected in only (10%) of isolates. Meanwhile, sulfhydryl reagent variable β-lactamase gene (*bla-shv*), which degrades narrow-spectrum cephalosporins as well as penicillin G, ampicillin, antipseudomonal penicillin, was not confirmed during this assay (Figure 5).

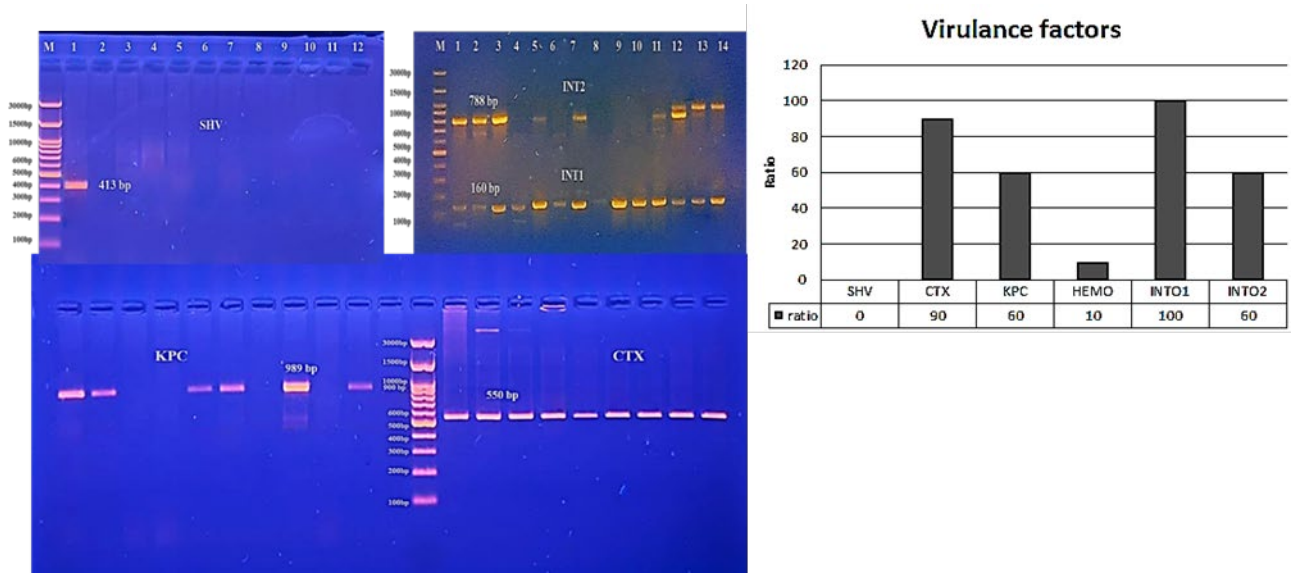

Figure 5 – PCR amplifectoin of virulence genes in *K. pneumoniae.* It shows PCR products of the subjected genes with particular sizes on agarose gel using two types of DNA marker (3kb) according to the size of amplicon. The diagram shows the ratio of detected genes in *K. pneumoniae* isolates.

9. Discussion

In this study, 55% of total fecal samples had the presence of bacteria determined as *K. pneumoniae* based on the growth on Orientation Chrome media. Although this media has a deferential function and helps to obtain a probable identification, it still cannot give a 100% guarantee to determine bacteria species. This uncertainty could depend on the genetic features and the source of the isolates (Lengfelder et al., 2019). However, some well-developed laboratories use bacteria as a part of growing other organisms, such as amoeba (Alsaadawi et al., 2019).

Subsequently, the detection was confirmed by PCR, which confirmed that 82% of the total suspected samples were *K. pneumoniae*. These differences might happen for the following reasons: First, the colony morphology and biochemical features on Chrome agar could be similar to other bacteria. Second, the subjected bacteria might have obtained critical genetic mutations that made the 16S rRNA gene difficult to recognize by their specific primes. Moreover, a technical issue during DNA extraction or PCR sample preparation stages may happen (Table 2).

The current project identified isolates that are highly similar in nucleotide sequences to isolates from other areas. These similarities could be due to an evolutionary manner or drug selection for *K. pneumoniae* strains in Iraqi camels, as well as the possibility of the presence of unexplored reservoirs that transported this opportunistic pathogen around the world. Moreover, the antimicrobial resistance (AMR)-encoding genes that are carried naturally on several plasmids for expressing MDR phenotypes in these bacteria are considered a main challenge to human health, animals, and the environment (Hu et al., 2021).

During the antibiotic sensitivity test, *K. pneumoniae* isolates were highly sensitive to levofloxacin, trimethoprim, and ceftriaxone. Meanwhile, they showed some resistance to vancomycin (100%) and amoxicillin (75%). These results were different from what Molana (2011) verified that *K. pneumonia* strains isolated from clinical samples in Babol City were resistant to cefotaxime and ceftriaxone (40%), Amikacin, and imipenem (50%). The wildlife of camels with a good immune system and less exposure to antibiotics led *K. pneumoniae* to own an undeveloped antibiotic resistance system and be susceptible to a wide range of antibiotics in comparison to those isolated from humans and cattle, which were found to be responsible for ESBL producing *K. pneumoniae* transfer between cows to humans due to environmental handling or consumption contaminated meat (Aldabbagh, 2022).

More studies in bacterial resistance genes to β-lactamase are required. *bla-ctx* and *bla-shv* genes of positive ESBLs are the main elements of drug resistance bacteria to β-lactam antibiotics (Robledo et al., 2011). In this study, the detection percentage of(*bla-ctxM*) ESBLs in K. pneumoniae in camels was (90%) which is less than what Al-Dabbagh (2022) recorded (100%) and higher than the observed results in Iran (78.9%) and Beijing (84.8%) human isolates (Awari, Nighute and Khatoon, 2013). This refers to the regional impact of the increasing use of antibiotics. Contrariwise, the current study reported zero percentages of *bla-shv* ESBL-producing organisms. This was incompatible with Indian and Korean studies that reported 6.0 and 87.0% frequency of ESBL-producing *K. pneumoniae*, respectively (Robledo et al., 2011).

bla-shv class of enzymes is the progenitor in *K. pneumoniae* and up to 20% of the plasmid-mediated ampicillin resistance under its control. Additionally, the *bla-shv* -1 β-lactamase gene might develop as a plasmid or chromosomal gene in *K. pneumoniae* (Asghari et al., 2021). The results also showed no detection of the Hemolysis gene (hly) in all *K. pneumoniae* isolates, which is exactly compatible with what Kuş (2023) mentioned in their study. Moreover, the rate of KPC gene detection in this study was (60%), which was a bit lower rate than what Robledo (2011) revealed (73%) (Firoozeh et al., 2019).

Altogether, *K. pneumoniae* isolates were positive for integron class 1. This was similar to Asghari et al. (2021). The high rate of integron class 1 might be due to the relationship between integron I and the occurrence of multidrug-resistant gram-negative bacteria (Li et al., 2013). Meanwhile, the prevalence of class 2 integrons in our MDR *K. pneumoniae* isolates was 60%, which is higher than Asghari et al. (2021) and Firoozeh et al. (2019).

10. Conclusion

This study illustrated that *K. pneumoniae* was responsible for the majority of respiratory and JIT infection in camels. In addition, this pathogen appeared to have low resistance toward the tested antibiotics with variable frequencies of virulence factor genes.

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