

# Detection of Several Carbapenems Resistant and Virulence Genes in Classical and Hyper-virulent Strains of *Klebsiella pneumoniae* Isolated from Hospitalized Neonates and Adults in Khartoum

Aalaa Mahgoub Albasha (✉ [loleta95227@outlook.com](mailto:loleta95227@outlook.com))

Sudan University of Science and Technology <https://orcid.org/0000-0002-9501-7264>

Esraa Hassan Osman

Sudan University of Science and Technology

Saga Abd Alhalim

Sudan university of science and technology

Elianz Alshaib

Sudan University of Science and Technology

Leena Al-Hassan

Brighton and Sussex Medical School

Hisham N Altayb

King Abdulaziz University

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## Research

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## Abstract

## Background

Carbapenems resistant hypervirulent strains of *Klebsiella pneumoniae* are one of the most critical organisms that cause fatal nosocomial infections. This study aimed to detect and characterize *K. pneumoniae* virulence genes (*mrkD*, *entB*, *rmpA*, *K2*, *kfu*, and *magA*) and carbapenem resistant (*bla<sub>NDM</sub>*, *bla<sub>IMP</sub>*, *bla<sub>OXA-48</sub>*, and *bla<sub>KPC</sub>*) genes

## Methods

Sixty *K. pneumoniae* strains were isolated from urine, blood, wound swab, and sputum samples, in two age groups: neonates and adults. String test was used to detect hypervirulent strains. Susceptibility testing for a wide range of antibiotics was performed on all isolates. DNA was extracted by the guanidine chloride method, then multiplex PCR was used for the detection of carbapenem-resistance and virulence genes.

## Results

Seventy percent of the isolates were resistant to ceftazidime and 8% to imipenem, 35% were multi-drug resistant, and 7% extensively drug-resistant, all neonatal blood isolates (n = 15) were resistant to ceftazidime. *entB* was the most predominant virulence gene (93.3%), followed by *mrkD* (78.3%), *kfu* (60%), *K2* (51.7%), *magA* (18.3%) and *rmpA* (5%). *bla<sub>OXA-48</sub>* was the most predominant carbapenem-resistant gene (68.3%), followed by *bla<sub>NDM</sub>* (10%), *bla<sub>KPC</sub>* (8.3%), and *bla<sub>IMP</sub>* (3.3%). Eight hyper-virulent strains were positive for *bla<sub>OXA-48</sub>* and two for *bla<sub>NDM</sub>* genes. The study concluded that there is a high rate of carbapenems resistant genes in hyper-virulent strains of *K. pneumoniae* isolated from hospitalized patients.

## Conclusion

The study reported for the first time in Sudan presence of carbapenems resistant genes in hyper-virulent strains of *K. pneumoniae* isolated from hospitalized patients. Presence of MDR and XDR strains of *K. pneumoniae* in neonatal ward in some Sudanese hospitals.

## Background

*Klebsiella pneumoniae* (*K. pneumoniae*) is a non-motile, capsulated gram-negative rod about 1–2 µm long, and is a facultative anaerobe [1]. It is a common cause of urinary tract, soft-tissue, and central nervous system infections, in addition to endocarditis, and cases of severe bronchopneumonia, sometimes with chronic destructive lesions and multiple abscess formation in the lungs. In many cases, localized infections lead to bacteremia [1].

There are two types of *K. pneumoniae* strains “classic” (cKp), usually non-virulent and drug-resistant gene producer and usually associated with hospital infections, while the other type is a hypervirulent (hvKp) drug-sensitive strain [2]. *K. pneumoniae* possesses different virulence and antimicrobial resistance genes associated with various clinical conditions. The vast majority of *K. pneumoniae* strains produce a capsular polysaccharide (CPS), which is considered the most crucial virulence factor [3], and its expression is enhanced by the regulator of mucoid phenotype A (*rmpA*) gene, which has an association with community-acquired pyogenic liver abscess (CA-PLA) [4]. *Klebsiella* ferric uptake (*kfu*) gene is a putative pathogenic gene associated with purulent tissue infections, capsule formation, and virulent hypermucoviscosity phenotype [5], mucoviscosity-associated gene-A (*magA*) in *K. pneumoniae* is associated with pyogenic liver abscess [6]. Most strains of *K. pneumoniae* have K markers; K1 and K2 were found to be significantly more resistant to phagocytosis and associated with nosocomial infection than non-K1/K2 isolates [7]. *mrkD* (mannose resistant *Klebsiella* like hemagglutinin-D) gene mediates binding to extracellular matrices and likely to have a role in the colonization of damaged epithelial surfaces [8]. Enterobactin-B (*entB*) is considered as one of the siderophore systems, which has a high affinity to bind to Fe [9].

Carbapenemase enzymes of *K. pneumoniae* can resist most  $\beta$ -lactam-ring-containing antibiotics, including carbapenems, and thus conferring resistance to these drugs [10]. Ambler molecular class A *K. pneumoniae* carbapenemase (KPC), class B; Verona integron metallo-beta-lactamases types (VIM), Imipenemase (IMP) and New Delhi metallo-beta-lactamase (NDM) and class D oxacillinase-48 (OXA-48) are frequently isolated from severe hospital infections [11]

Carbapenem resistant hypervirulent strains of *K. pneumoniae* are one of the most important organisms that cause fatal nosocomial infections [12]. Recently, increasing reports of resistance to carbapenem in healthcare-associated with *K. pneumoniae* infections have been documented in Sudan [13] [14] [15]. The mortality rate of carbapenem-resistant *K. pneumoniae* bacteremia could reach 50% of cases [16].

However, up to date, there is no published data in Sudan about the distribution and epidemiology of various types of Carbapenemases and virulence genes on hvKp and cKp strains circulating in Khartoum hospitals. This information is of great importance to understand their local epidemiology and to establish eradication and prevention procedures. Thus this study was conducted to detect and to characterize the common virulence and carbapenem-resistant genes of hvKp and cKp strains isolated from hospitalized patients in different hospitals in Khartoum state.

## Results

### Demographic data

Sixty *K. pneumoniae* isolates were obtained from different hospitals in Khartoum State, 27 (45%) were from females, and 33 (55%) from males, 37 (61.7%) were from urine, 15 (25%) were from neonatal sepsis, and 11 (18%) were from elderly patients (Table 2).

### String test

Out of sixty *K. pneumoniae* isolates, 9 (16%) were hypermucoviscous, and 47 (84%) isolates were classic.

### Susceptibility test results

Most strains, 42 (70%), were resistant to ceftazidime and only 5(8%) resistant to imipenem (Table 2). Multi-drug resistant isolates were detected in 12 of urine isolates, 7 of blood, and 2 of wound swab isolates. Three neonatal blood isolates and one adult wound swab were showed extensively drug-resistant.

### PCR results

#### Detection of *K. pneumoniae* carbapenem-resistant and virulence genes

Eighty percent (48/60) of isolates were positive for carbapenem-resistant genes: 68.3% (41/60) were positive for *bla*<sub>OXA-48</sub> gene, 10% (6/60) were positive for *bla*<sub>NDM</sub> gene, 8.4% (5/60) were positive for *bla*<sub>KPC</sub> gene, and 3.3% (2/60) were positive for *bla*<sub>IMP</sub> gene. One neonatal blood isolate possesses three carbapenem-resistant genes (*bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub>, and *bla*<sub>IMP</sub>), six isolates possess two genes (four possess *bla*<sub>OXA-48</sub> and *bla*<sub>NDM</sub>, two possess *bla*<sub>OXA-48</sub> and *bla*<sub>KPC</sub>), and thirty-nine isolates possess one gene (34 *bla*<sub>OXA-48</sub>, two *bla*<sub>NDM</sub>, two *bla*<sub>KPC</sub>, and one *bla*<sub>IMP</sub>) and the remaining (12) were negative for all carbapenem-resistant genes. Eight hyper-virulent strains were harboring *bla*<sub>OXA-48</sub> and two harboring *bla*<sub>NDM</sub> genes (Table 4) (Figures 1, 2, and 3).

For virulence genes *mrkD* detected in 47 (78.3%) isolates, *entB* in 56(93.3%), *rmpA* in 3(5%), *K2* in 31(51.7%), *kfu* in 36 (60%) and *magA* in 8(13.3%) isolates. (Table 3) (Figure 4).

There was no significant statistical association between the presence of virulence genes and carbapenems resistant genes (*p*-value was <0.05). A total of 92% (43/47) of *mrkD* gene positive isolates were positive for one or more carbapenem resistant genes. There was strongly significant association between the presence of *mrkD* and *entb* genes (*p*-value = 0.0005), they were co-existed in 46 isolates.

## Discussion

In this study, eight hyper-virulent strains of *K. pneumoniae* were reported positive for carbapenems resistant genes (*OXA-48* and *NDM*). The presence of these strains in the clinical setting will complicate clinical practice and will cause fatal nosocomial infections [12]. Although antimicrobial-resistant hvKP strains are rarely reported worldwide [17–19], but here in Sudan they appear to be more prevalent.

Eight neonatal blood isolates were multidrug-resistant, and three of them were extensively resistant to all antibiotics that were used. Consequently the emergence of MDR pathogens would increase the mortality and morbidity and prolong hospitalization and cost of treatment [20].

All neonatal blood isolates (15) were resistant to ceftazidime. Ceftazidime-resistant *Klebsiella pneumoniae* (CRKP) in the pediatric oncology units of some Sudanese hospitals may be the cause of recent reports of high mortality rate associated with *K. pneumoniae* infections among this group in different Sudanese hospitals [21]. According to Schiappa [22], high resistance rates to ceftazidime could be due to the presence of a predominant enzyme (TEM-10) responsible for ceftazidime resistance in bloodstream isolates.

The isolates showed varying degrees of resistance to the other antibiotics; ciprofloxacin 30%, gentamicin 40%, and ceftazidime (70%). Resistance to these antibiotics may also be due to the presence of Extended-Spectrum Beta-lactamases (ESBLs) and other mechanisms like efflux pumps and porin mutations [23], which were not covered in this study.

Although chloramphenicol is used as a treatment of choice for MDR gram-negative bacilli bacteria [24], 38% of our isolates were resistant to it, which may be caused by transferable enzymatic resistance to aminoglycosides, that is common in some hospitals [25].

In the current study, 94% (51/54) of the isolates harboring carbapenem-resistant genes were phenotypically susceptible to imipenem. This confirms what Walsh [26] said that this gene is not stable and relies upon other synergistic mechanisms to mediate resistance against carbapenems. In addition to imipenem, other antibiotics were analyzed in this study.

Of 48 *K. pneumoniae* isolates detected of having carbapenem-resistant genes, 10 had multiple genes co-occurring. This finding agrees with Ali & Omer [27] and Satir [28], which showed a multiplicity of genes in their isolates.

A total of 80% (4/5) of *KPC* and 100% (2/2) of *IMP* genes were positive among infant blood samples, and this may be due to organisms harboring these genes have a high ability to cause systemic infections, particularly in immunocompromised patients [29].

In this study, we found the essential gene for *K. pneumoniae* siderophores system *entB* gene is positive in 93.3% of all *K. pneumoniae* isolates, the rest (6.7%) of isolates that do not possess *entB* may contain other enterobactin (*entA*, C, D, E or F), or other siderophores systems like yersiniabactin or aerobactin as reported by Lawlor [20]. Furthermore, *mrkD* gene is presented in 78.3% of the isolate. This gene has been found to be important in adhesion as reported by Chen et al. (2012)[29]. The *rmpA* gene was detected in 5% of isolates, in contrast with Aljanaby and Alhasani [20] who found the *rmpA* gene present in 62.5% of *K. pneumoniae* isolates. This difference may be attributed to its mode of inheritance as plasmid-mediated as mentioned by [20] indicating the limited spread of this gene in our local strains in Sudan.

The capsular serotype gene *K2* was present in 51.7% of isolates; the rest of isolates may contain other capsular serotypes, as mentioned by Ho [30]. This study showed that *K2* is present in 80% of hypermucoviscous strains, indicating that there is a relationship between the presence of *K2* gene and hypermucoviscous strains of *K. pneumoniae*, which is in agreement with the study by Guo [31] which found that *K2* is the most common capsular serotype in hypermucoviscous strain. In contrast to other studies [20, 32] [33, 34], which found *K1* was the most prevalent capsular serotype among hypermucoviscous *K. pneumoniae*.

The *kfu* gene (which codes for an iron uptake system) was present in 60% of isolates. The study showed no association between the presence of *kfu* gene and hypermucoviscosity. This finding disagrees with previous studies [20, 35, 36], which showed that *kfu* gene is associated with hypermucoviscosity phenotype; which may be attributed to diversity in geographical locations of studies.

The *magA* gene was found in 13.3% of isolates. The study showed no association between the presence of *magA* gene and hypermucoviscous strains. Although this gene is highly essential for *K. pneumoniae*, which confirms bacterial mucoviscosity, its prevalence among local isolates is not high, suggesting that other genes play a role in the formation of mucoviscosity [20].

## Conclusion

The study reported for the first time in Sudan presence of carbapenems resistant genes in hyper-virulent strains of *K. pneumoniae* isolated from hospitalized patients. Presence of MDR and XDR strains of *K. pneumoniae* in neonatal ward in some Sudanese hospitals.

## Materials And Methods

A total of 60 isolates of *Klebsiella pneumoniae* were obtained from hospitalized patients in different hospitals in Khartoum state (Royal Care International Hospital, Al-Baraha Hospital, Soba University Hospital, Al-Amal National Hospital, East Nile Model Hospital, Omdurman Medical Military Hospital, and Police Hospital) during the period from January 2017 to March 2017 They were isolated from the urine (37), blood (14), respiratory tract (4) and wound infection (5).

### Bacterial identification

The isolates were identified by gram stain, standard biochemical methods (urease test, indole test, and carbohydrates fermentation test, motility test, and citrate utilization test) [37] [38], and by *K. pneumoniae* species-specific primers (Table 1) targeting the 16S rRNA gene. Antibiotic susceptibility testing was done by the Kirby Bauer disc diffusion method on Mueller Hinton agar using the following antibiotics; ciprofloxacin (5mcg), gentamicin (10mcg), ceftazidime (30mcg), imipenem (10mcg), and chloramphenicol (30) (HiMedia Laboratories Pvt. Ltd. Mumbai, India) [39]. *E. coli* ATCC 25922 and *K. pneumoniae* (ATCC 700603) were used as quality control strains.

Capsule stain was used to detect capsule [40]. String test was used to differentiate between hvKp and cKp strains: if the grown colonies of *K. pneumoniae* form a string >5 mm in length using a sterile loop, this demonstrates the hypermucoviscosity phenotype [41].

### DNA extraction

DNA was extracted using the guanidine chloride method [42]. Several colonies from overnight nutrient agar growth were washed with normal saline (NS), followed by the addition of 2 mL lysis buffer, 5µl proteinase K, 1ml guanidine chloride, and 300 µl ammonium acetate. The suspension was incubated overnight at 37°C, on the next day, 2ml of chilled chloroform was added. After centrifugation, the transparent upper layer was collected in a new 15ml tube, then 10 ml of absolute cold ethanol was added to enhance precipitation of DNA. The pellet was washed with 70% ethanol and allowed to dry. The pellet was suspended with nuclease-free water and quantified using gel electrophoresis. The DNA samples were stored at -80°C until used for PCR.

### Detection of virulent and resistant genes

A primers sets targeting virulence and carbapenem-resistant genes of *K. pneumoniae* are shown in table 1. The primers were dissolved according to manufacturer guidelines to prepare 10pmol/µl in all PCR reactions.

### PCR conditions

PCR was carried out in a 20µl volume using the Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea), 1µl of each forward and reverse primer (10 pmol/µL), 2 µl of DNA, and then the volume was completed to 20 µl by distilled water. Four multiplex and single reaction PCR protocols were used for amplification of 16S rRNA, resistant and virulence genes, the initial melting temperature for all was 95°C for 5 minutes and a final extension was at 72°C for 10 minutes. Details of annealing temperatures are listed in Table 1.

### Data analysis:

Data were analyzed by Statistical package for the social sciences (SPSS) version 16. Chi-square test was used for the analysis of the relationship between variables. A *p*-value of <0.05 was considered statistically significant

## Abbreviation

**bla:** β-lactamase

**CA-PLA:** community-acquired pyogenic liver abscess

**cKp:** classic *K. pneumoniae*

**CLSI:** Clinical and Laboratory Standards Institute

**CPS:** capsular polysaccharide

**entB:** Enterobactin B

**ESBL:** extended- spectrum  $\beta$ - lactamase

**hvKp:** Hyper-virulent *Klebsiella pneumoniae*

**IPM:** Imipenem

**kfu:** Klebsiella Ferric Uptake

**KPC:** *Klebsiella pneumoniae* carbapenemase

**OXA-48:** Oxacillinase 48

**magA:** Mucoviscosity-Associated Gene A

**MDR:** Multi Drug Resistant

**mrkD:** Mannose Resistant Klebsiella like hemoagglutinin D

**NDM:** New Delhi metallo

**PCR:** polymerase chain reaction

**mpA:** Regulatory of Mucoïd Phenotype A

**SPSS:** Statistical Package for the Social Sciences

**XDR:** Extensively drug-resistant

## Declarations

### Ethics approval and consent to participate

The research was approved by institutional ethics committee of deanship of scientific research, Sudan University of Science and Technology No: DSR-IEC3-01-07.

### Consent for publication

Not applicable.

### Availability of data and materials

Data is available from corresponding author upon request

### Competing interests

The authors declare that they have no competing interests.

### Funding

None

## Authors' contributions

AMA, HNA, SAA, EFA and EHO designed the study, AMA, SAA, EFA and EHO performed the experiments, HNA, AMA, and SAA analyzed the data, HNA, AMA and LAH wrote the manuscript, all the authors approved the final version of the manuscript.

## Acknowledgements

Not applicable

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## Tables

Table 1 Primers sequences and PCR protocols used in this study

Protocols	Temperature cycling	Marker	Sequence (5-3')	Amplicons size (bp)	References
1 <sup>st</sup>	35 cycles at 94°C for 30s, 58°C for 90s and 72°C for 90s	16s rRNA	F. ATTTGAAGAGGTTGCAAACGAT R. TTCACTCTGAATTTTCTTGTTTC	130	[43]
2 <sup>nd</sup>	30 cycles at 94°C for 30s, 60°C for 45s, and 72°C for 60s	<i>mrkD</i>	F. AAGCTATCGCTGTACTTCCGGCA R. GCGTTGGCGCTCAGATAGG	340	* [44]
		<i>entB</i>	F. GTCAACTGGGCCTTTGAGCCGTC R. TATGGGCGTAAACGCCGGTGAT	400	
		<i>rmpA</i>	F. CATAAGAGTATTGGTTGACAG R. CTTGCATGAGCCATCTTCA	461	
		<i>K2</i>	F. CAACCATGGTGGTCGATTAG R. TGGTAGCCATATCCCTTTGG	531	
		<i>kfu</i>	F. GGCCTTTGTCCAGAGCTACG R. GGGTCTGGCGCAGAGTATGC	638	
		<i>magA</i>	F. GGTGCTCTTTACATCATTGC R. GCAATGGCCATTGCGTTAG	1283	
3 <sup>rd</sup>	35 cycles at 94°C for 20s, 56°C for 10s, 72°C for 20s	<i>NDM</i>	F. GGTTGGCGATCTGGTTTTC R. CGGAATGGCTCATCACGATC	521	[45][45][45][45][25] [25]
		<i>IMP</i>	F. TTGACACTCCATTACAG R. GATTGAGAATTAAGCCACTCT	232	
4 <sup>th</sup>	35 cycles at 94°C for 45s, 52°C for 1 minute, and 72°C for 1 minute	<i>KPC</i>	F. CATTCAAGGGCTTTCTTGCTGC R. ACGACGGCATAGTCATTGTC	498	
		<i>OXA-48</i>	F. GCTTGATCGCCCTCGATT R. GATTTGCTCCGTGGCCGAAA	281	

Abbreviations: s= second, F= Forward, R= Reverse, bp= base pair

\*Annealing time changed from 90s to 45s.

Table (2) Susceptibility testing profile of *K. pneumoniae* strains among different clinical specimens and age groups.

		ciprofloxacin		chloramphenicol		gentamicin		imipenem		ceftazidime	
		sensitive	resistant	sensitive	resistant	sensitive	resistant	sensitive	resistant	sensitive	resistant
Sex	male	20 (48%)	13 (72%)	17 (46%)	16 (70%)	17 (47%)	16 (67%)	31 (56%)	2 (40%)	10 (56%)	23 (55%)
	female	22 (52%)	5 (28%)	20 (54%)	7 (30%)	19 (53%)	8 (33%)	24 (44%)	3 (60%)	8 (44%)	19 (45%)
	<i>p</i>	0.082		0.076		0.143		0.49		0.95	
Sample	urine	27 (64%)	10 (56%)	24 (65%)	13 (57%)	24 (67%)	13 (54%)	37 (67%)	0 (0%)	15 (83%)	22 (52%)
	blood	8 (19%)	6 (33%)	7 (19%)	7 (30%)	6 (17%)	8 (33%)	10 (18%)	4 (80%)	0 (0%)	14 (33%)
	wound swab	3 (7%)	2 (11%)	3 (8%)	2 (9%)	3 (8%)	2 (8%)	4 (7%)	1 (20%)	1 (6%)	4 (10%)
	sputum	4 (10%)	0 (0%)	3 (8%)	1 (4%)	3 (8%)	1 (4%)	4 (7%)	0 (0%)	2 (11%)	2 (5%)
	<i>p</i>	0.80		0.95		0.83		0.23		0.38	
Total		42	18	37	23	36	24	55	5	18	42

*P*= *p*value, N= number, \* =significant

Table 3: The association between *K. pneumoniae* virulence genes production with sample type and mucus production

Sample	<i>entB</i>		<i>rmpA</i>		<i>k2</i>		<i>kfu</i>		<i>magA</i>		<i>mrkD</i>	
	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative	positive	negative
Urine	34 (61%)	3 (75%)	2 (67%)	35 (61%)	21 (68%)	16 (55%)	24 (67%)	13 (54%)	6 (75%)	31 (60%)	30 (64%)	7 (54%)
Blood	14 (25%)	0 (0%)	0 (0%)	14 (25%)	5 (16%)	9 (31%)	6 (17%)	8 (33%)	2 (25%)	12 (23%)	12 (26%)	2 (15%)
Wound swab	5 (9%)	0 (0%)	0 (0%)	5 (9%)	2 (6%)	3 (10%)	4 (11%)	1 (4%)	0 (0%)	5 (10%)	4 (9%)	1 (8%)
Sputum	3 (5%)	1 (25%)	1 (33%)	3 (5%)	3 (10%)	1 (3%)	2 (6%)	2 (8%)	0 (0%)	4 (8%)	1 (2%)	3 (23%)
<i>p</i>	0.73		0.43		0.86		0.86		0.24		0.07	
M Hyper	9 (16%)	1 (25%)	0 (0%)	10 (18%)	8 (26%)	2 (7%)	4 (11%)	6 (25%)	1 (13%)	9 (17%)	10 (21%)	0 (0%)
M Classic	47 (84%)	3 (75%)	3 (100%)	47 (82%)	23 (74%)	27 (93%)	32 (89%)	18 (75%)	7 (88%)	43 (83%)	37 (79%)	13 (100%)
<i>p</i>	0.64		0.42		0.050		0.15		0.73		0.06	
Total	56	4	3	57	31	29	36	24	8	52	47	13

*P*= *p*-value, M= Mucoviscosity

Table 4: The association between imipenem susceptibility testing, mucoviscosity, and carbapenems resistant genes

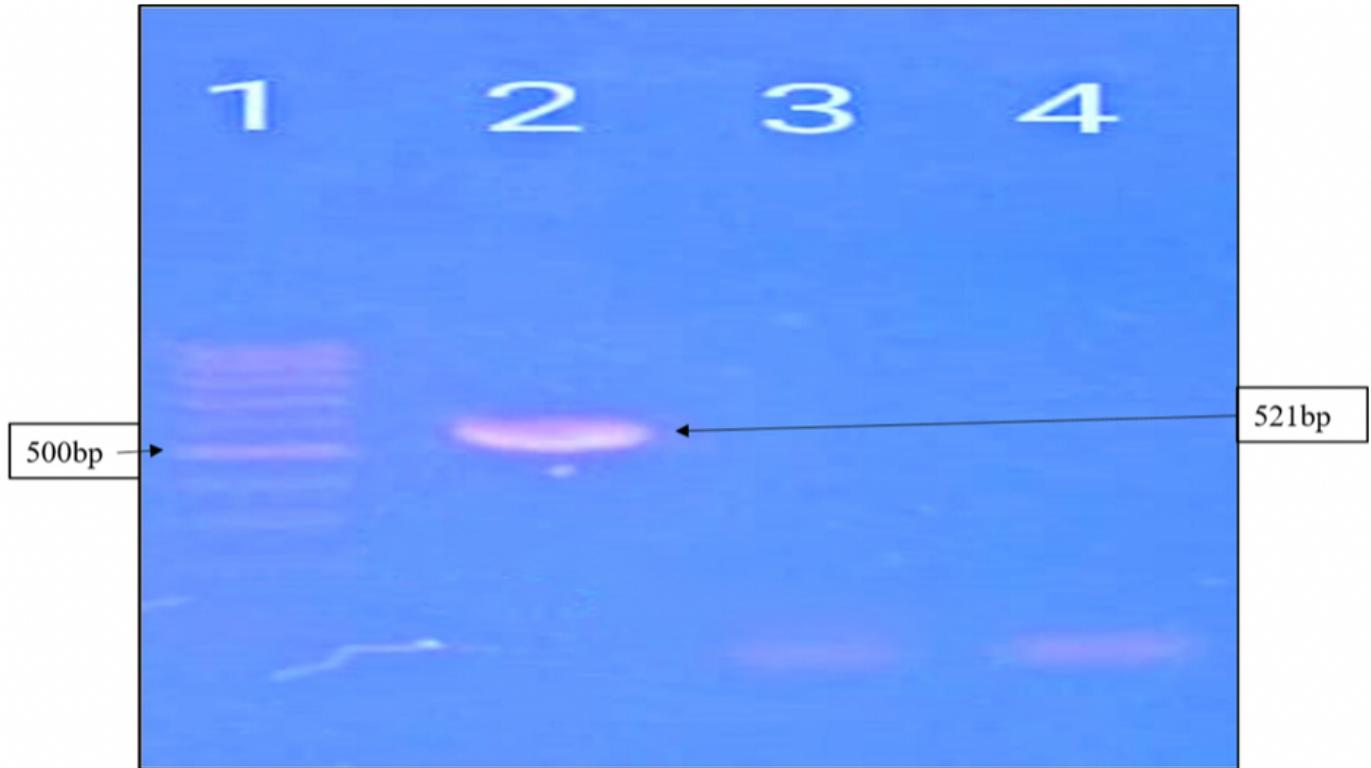
		<i>IMP</i>		<i>OXA-48</i>		<i>KPC</i>		<i>NDM</i>	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Imipenem	S	2 (4%)	53 (96%)	38 (69%)	17 (31%)	5 (9%)	50 (91%)	6 (11%)	49 (89%)
	R	0 (0%)	5 (100%)	3 (60%)	2 (40%)	0 (0%)	5 (100%)	0 (0%)	5 (100%)
<i>p</i>		0.76		0.57		0.03*		0.63	
Mucoviscosity	H	0 (0%)	10 (100%)	8 (80%)	2 (20%)	0 (0%)	10 (100%)	2 (20%)	8 (80%)
	C	2 (4%)	48 (96%)	33 (66%)	17 (34%)	5 (10%)	45 (90%)	4 (8%)	46 (92%)
<i>p</i>		0.52		0.39		0.30		0.26	
Total		2	58	41	19	5	55	6	48

Abbreviations: *P*=*p*-value, *R*=Resistant, and *S*= Sensitive, *H*= Hypermucoid, *C*=Classic

Table 5: The association between *K. pneumoniae* virulence and carbapenems resistant genes production

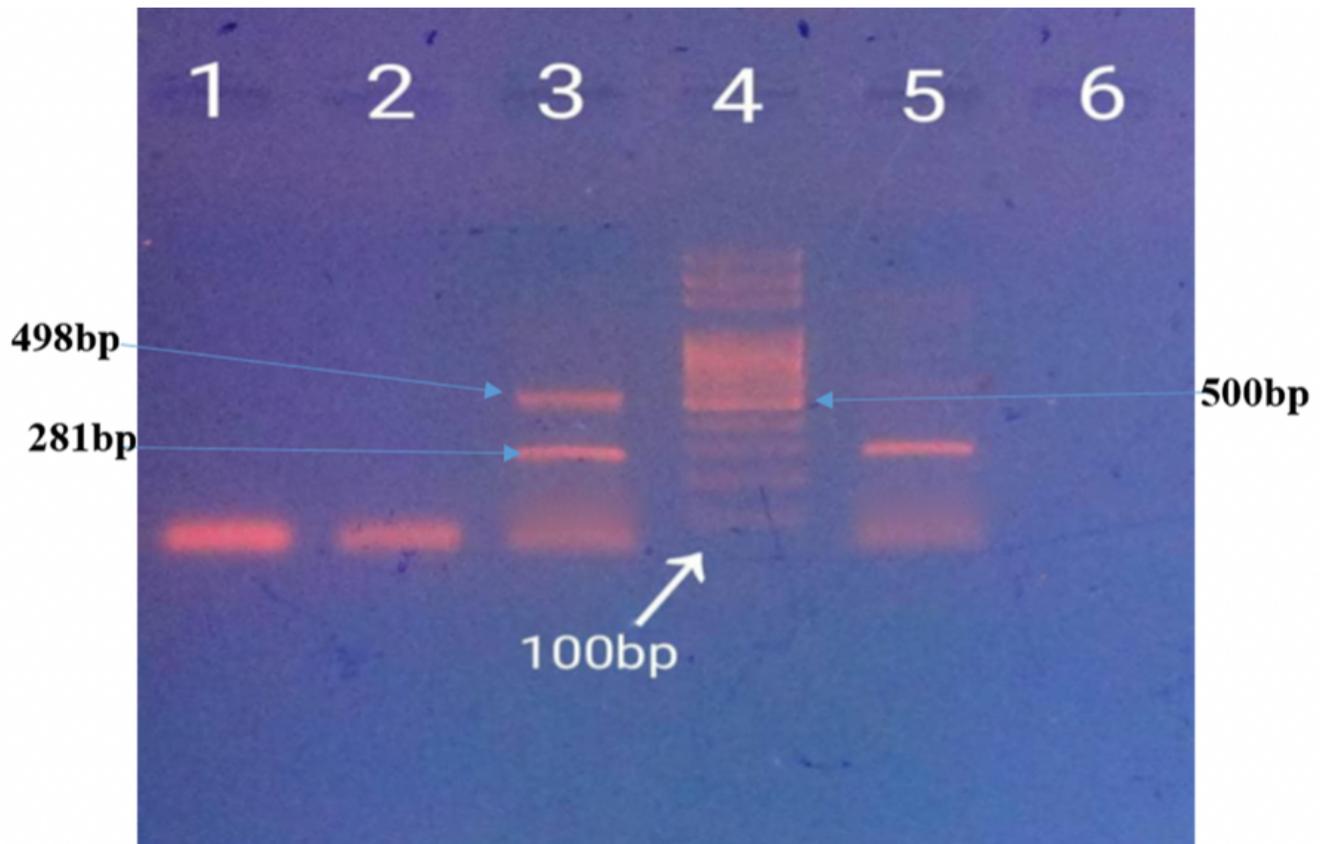
		<i>IMP</i>		<i>OXA-48</i>		<i>KPC</i>		<i>NDM</i>	
		positive	negative	positive	negative	positive	negative	positive	negative
<i>mrkD</i>	positive	1 (2)	46 (98%)	32 (68%)	15 (32%)	4 (9%)	43 (91%)	4 (9%)	43 (91%)
	negative	1 (8%)	12 (92%)	9 (69%)	4 (31%)	1 (8%)	12 (92%)	2 (15%)	11 (85%)
<i>p</i>		0.33		0.93		0.92		0.47	
<i>entB</i>	positive	2 (4%)	54 (96%)	39 (70%)	17 (30%)	5 (9%)	51 (91%)	4 (7%)	52 (93%)
	negative	0 (0%)	4 (100%)	2 (50%)	2 (50%)	0 (0%)	4 (100%)	2 (50%)	2 (50%)
<i>p</i>		0.70		0.42		0.51		0.005	
<i>rmpA</i>	positive	0 (0%)	3 (100%)	1 (33%)	2 (67%)	0 (0%)	3 (100%)	0 (0%)	3 (100%)
	negative	2 (4%)	55 (96%)	40 (70%)	17 (30%)	5 (9%)	52 (91%)	6 (11%)	51 (89%)
<i>p</i>		0.74		0.18		0.59		0.51	
<i>k2</i>	positive	1 (3%)	30 (97%)	21 (68%)	10 (32%)	1 (3%)	30 (97%)	3 (10%)	28 (90%)
	negative	1 (3%)	28 (97%)	20 (69%)	9 (31%)	4 (14%)	25 (86%)	3 (10%)	26 (90%)
<i>p</i>		0.94		0.92		0.14		0.93	
<i>kfu</i>	positive	0 (0%)	36 (100%)	26 (72%)	10 (28%)	1 (3%)	35 (97%)	3 (8%)	33 (92%)
	negative	2 (8%)	22 (92%)	15 (63%)	9 (38%)	4 (17%)	20 (83%)	3 (13%)	21 (88%)
<i>p</i>		0.08		0.43		0.05		0.60	
<i>magA</i>	positive	1 (13%)	7 (88%)	7 (88%)	1 (13%)	1 (13%)	7 (88%)	1 (13%)	7 (88%)
	negative	1 (2%)	51 (98%)	34 (65%)	18 (35%)	4 (8%)	48 (92%)	5 (10%)	47 (90%)
<i>p</i>		0.12		0.21		0.65		0.80	

## Figures



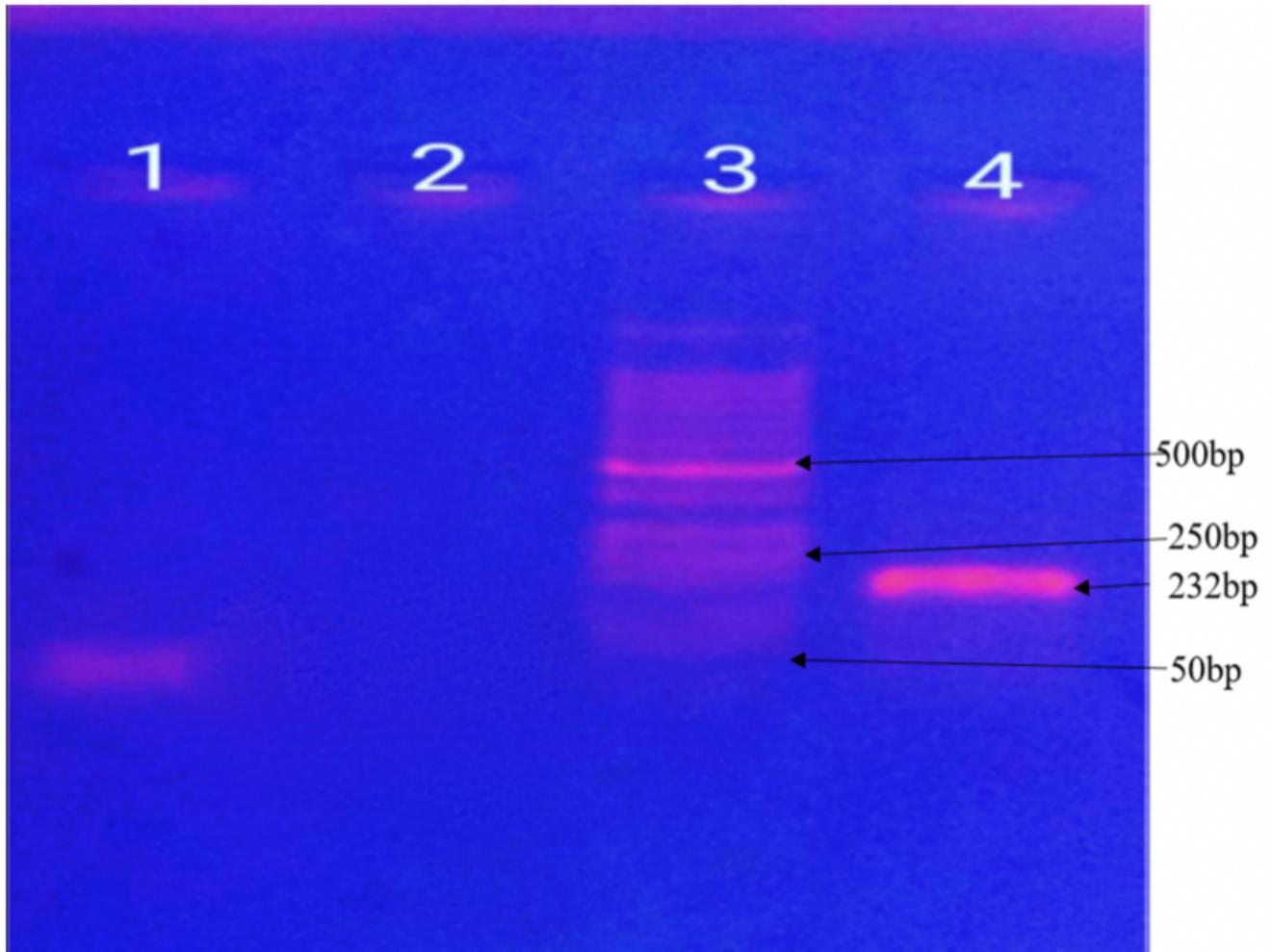
**Figure 1**

Amplified DNA of blaNDM gene. Lane 1, DNA ladder 100-1500bp; Lane 2, typical band size of 521bp corresponding to the molecular size of blaNDM gene; Lane 3 and 4, negative samples.



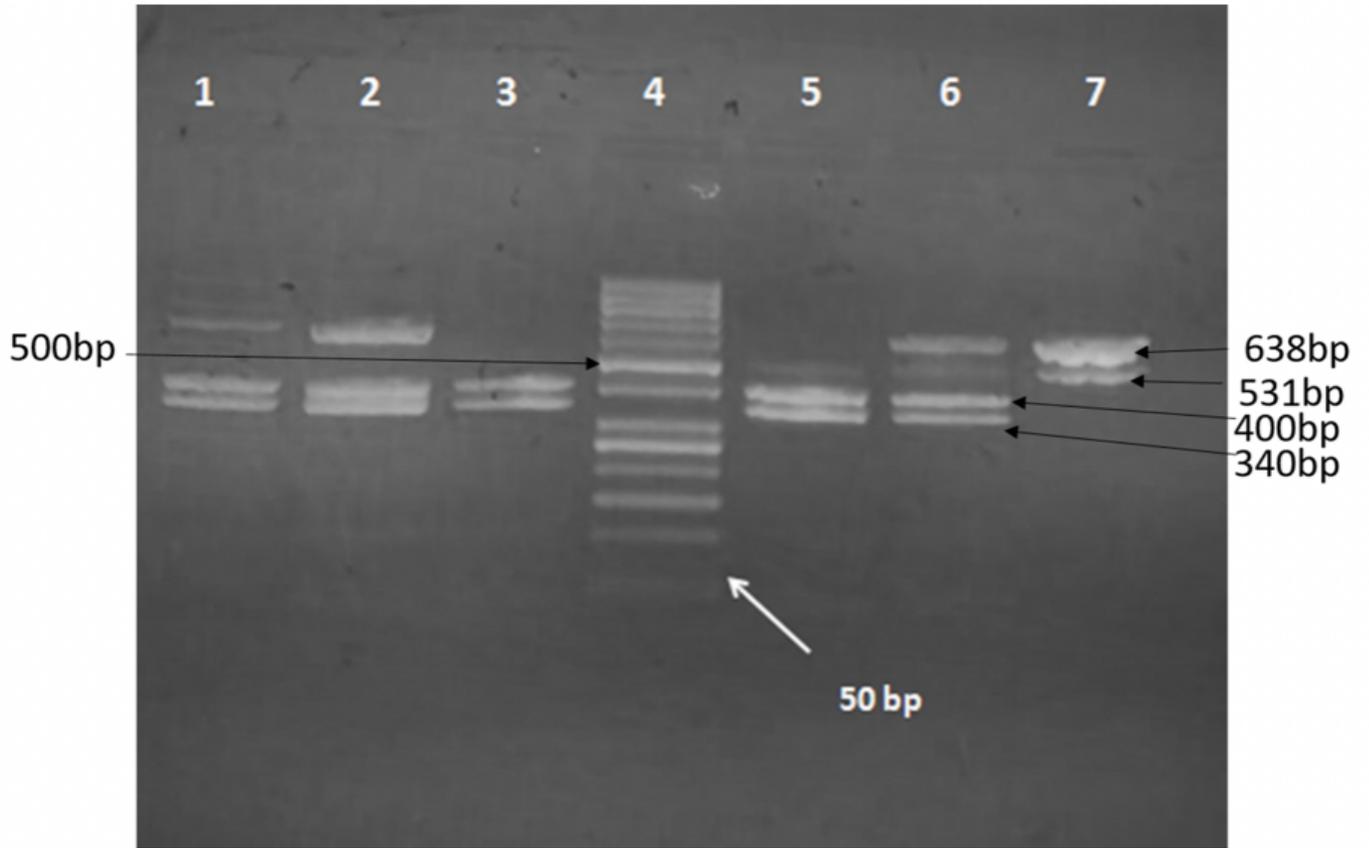
**Figure 2**

Amplified DNA of blaKPC and blaOXA-48 genes. Lane 4, DNA ladder 100-1500bp; Lane 3, typical bands size of 498bp and 281bp corresponding to the molecular size of blaKPC and blaOXA-48 genes respectively; Lane 5, typical band size of 281bp corresponding to the molecular size of blaOXA-48 gene; Lanes 1,2, and 6 are negative samples.



**Figure 3**

Amplified DNA of blaIMP gene. Lane 3, DNA ladder 50bp; Lane 4, positive sample corresponding to the molecular size of blaIMP gene; Lanes 1 and 2 are negative samples.



**Figure 4**

Multiplex PCR for amplification of *K. pneumoniae* virulence genes on 1.5% agarose gel electrophoresis. Lane 3 marker: 50 – 1000bp fragments. Lane 1 positive sample contain mrkD (340bp), entB (400bp) and kfu (638bp) genes. Lane 2 positive sample for mrkD and entB genes. Lane 4 positive sample for mrkD, entB, and K2 (531bp) genes. Lane 5 positive sample for mrkD, entB, K2 and kfu genes. Lane 6 positive sample for K2 and kfu genes.