

Phenotypic and Molecular Evaluation of *Klebsiella pneumoniae* in Traumatic Wounds: Prevalence, Antibiotic Resistance, and Risk Factors

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ABSTRACT

Klebsiella pneumoniae is a significant pathogen responsible for wound infections, particularly in traumatic injuries like crushing and laceration wounds. To determine the prevalence, antibiotic resistance patterns, and associated risk factors of *K. pneumoniae* infection in crushing and laceration wounds. A total of 166 samples; (95) samples from crush wounds and (71) samples from lacerations. Microbial isolation and antibiotic susceptibility testing, along with genetic analyses for (*aph (3')-Ia* and *aac(3)-II*) genes were performed to *K. pneumoniae* and its resistance patterns. Demographics, clinical details, and risk factors were documented for all patients. A total of 166 bacterial isolates were collected from patients; forty-nine isolates were *K. pneumoniae* (29.5%). The prevalence of crush wound samples was higher (37%) than lacerations (20%). Males represent (81.6%), with an average age of 41 years. Diabetic patients were (24.5%), while patients who were taking antibiotics before infection were (44.9%). ESBL genes and their resistance (*aph (3')-Ia* and *aac (3)-II*) were commonly found, which shows the presence of multiple drug resistance. The study reveals the widespread prevalence of *K. pneumoniae* and significant antibiotic resistance in crush and laceration wounds, particularly those associated with ESBL production. These findings highlight the need for effective infection control strategies, tailored treatments to limit spread, and close monitoring of antibiotic resistance patterns.

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1- INTRODUCTION

Klebsiella pneumoniae is an opportunistic Gram-negative bacterium widely associated with serious infections in traumatic injuries and hospitalized patients. Crush patients are susceptible to deep tissue damage and muscle tears, with motor vehicle and work-related accidents accounting for the largest proportion of these occurring in contaminated environments that facilitate the colonization of pathogens [1, 2].

Over the past decade, the emergence and spread of extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* strains has significantly reduced the effectiveness of commonly used beta-lactam antibiotics, including penicillin's and cephalosporins. Resistance is currently increasing at dangerous rates not only to first-generation cephalosporin antibiotics, but it has become a challenge to third-generation antibiotics such as (ceftazidime, cefotaxime, and ceftriaxone) and in some cases has reached fourth-generation options such as cefepime [3, 4].

Consequently, resorting to carbapenem antibiotics, such as imipenem, became necessary, as they were a last resort due to their demonstrated efficacy and low resistance rates [5, 6]. Aminoglycosides represent an important option for the management of multidrug-resistant *K. pneumoniae* infections, particularly kanamycin and gentamicin, and are essential components of combination therapy. However, the high incidence of aminoglycoside resistance is becoming increasingly apparent due to the bacterial acquisition of aminoglycoside-modifying enzyme genes. *aac* (3)-II encodes a 3-N-acetyltransferase, which confers resistance to gentamicin, and *aph* (3')-Ia encodes a 3'-phosphotransferase, which is also involved in resistance to the antibiotic kanamycin. These genes are transferring horizontally and are carry by plasmids, which explains their rapid spread in clinical settings [7, 8]. The coexistence of ESBL production and aminoglycoside resistance in *K. pneumoniae* presents a complex clinical challenge, as pathogenic isolates that exhibit both resistance traits are often resistant to antibiotics. This calls for serious intervention, including early and rapid detection and appropriate infection control measures [9, 10].

2- MATERIALS AND METHODS

2.1 Samples collection

Samples were collected 166 from patients admitted to hospitals in Diyala Governorate, Iraq, who had sustained injuries from accidents involving crush trauma and lacerations, such as car accidents, work accidents, and falls from heights, during the period from February to December 2024. Used a sterile cotton swab; pre-moistened with sterile saline to improve recovery of microorganisms [11]. Samples were transported to the microbiology laboratory for examination and evaluation at the laboratories of Yarmouk University College.

2.2 Culture and Identification

To isolate *K. pneumoniae*, wound samples were incubated aerobically for a whole night at 37°C, bacterial isolates were selected and purified and their colony characteristics, e.g., morphology, color and texture, was examined. The identification of isolation through morphological characteristics and performing biochemical tests, such as catalase, urease, oxidase, and Simons' citrate, was guided according to [12].

2.3 Antibiotic Sensitivity Testing Using the Mueller-Hinton Method

To decide which antibiotics would be effective against the *K. pneumoniae* isolates, we used the Mueller-Hinton agar disk diffusion method follows the standards set by the Clinical and Laboratory Standards Institute [13]. First, colonies from each bacterial isolate were suspended in sterile saline to match a 0.5 McFarland turbidity standard this ensures a consistent number of bacteria are evaluated each time. Using a sterile cotton swab, the suspension was evenly spread across the surface of a Mueller- Hinton agar plate, creating a thin layer of bacteria ready for testing. Small antibiotic impregnated discs were placed onto the surface of the plate. Common antibiotics included Cephalosporins Antibiotic tests, First-Generation (Cephalexin). Third-Generation Cefotaxime, Ceftazidime, Ceftriaxone. Fourth Generation (Cefepime) and included Carbapenem (Imipenem), kanamycin, Gentamicin. The plates were incubated at 37°C for 16 to 18 hours. After incubation, we looked for clear zones around the discs, where bacterial growth had been inhibited. The diameter of these zones was measured and compared to CLSI guidelines to decide whether the bacteria were susceptible, intermediate, or resistant to each antibiotic according to [13].

Table (1): Standard for *Klebsiella pneumoniae* by CLSI disk-diffusion practice

Antibiotic class	Drug	Standard disc content
First-gen cephalosporin	Cephalexin	30 µg
Third-gen cephalosporin	Cefotaxime ,Ceftazidime, Ceftriaxone	30 µg
Fourth-gen cephalosporin	Cefepime	30 µg
Carbapenem	Imipenem	10 µg.
Aminoglycoside	kanamycin	10 µg.
Aminoglycoside	Gentamicin	10 µg.

2.4 Aminoglycoside Genes Identification

1. Aminoglycoside Genes primers

Finding the *aac (3)-II* and *aph (3)-Ia* resistance genes in *K. pneumoniae* using PCR by previous study [14].

Table (2): Primers sequences and melting temperatures used for amplification

Primer Name	Seq.	Annealing Temp. (°C)	Product Size (bp)
II aac (3)-II-F	ATATCGCGATGCATACGCGG	56	877
II aac (3)-II-R	GACGGCCTCTAACCGGAAGG		
aph (30)-Ia-F	CGAGCATCAAATGAAACTGC	50	623
aph (30)-Ia-R	GCGTTGCCAATGATGTTACAG		

2. PCR Protocol

Table (3): PCR Component Calculation

No. of Reaction	15	rxn	Annealing temperature of primers	55,50
Reaction Volume /run	20	µl	Length of PCR product (bp)	877,623

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	10
Forward primer	10	µM	0.5	µM	1
Reverse primer	10	µM	0.5	µM	1
Nuclease Free Water					6
DNA		ng/µl		ng/µl	2
Total volume					20
Aliquot per single rxn	18µl of Master mix per tube and add 2µl of Template.				

Table (4): PCR Program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55 OR 50	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

3- RESULTS

Diagnostic tests were performed on 166 clinical wound samples, including 95 from patients with crushing injuries and 71 patients with laceration wounds. *K. pneumoniae* was isolated from 35/95, 37 % of crushing injuries, while 11 cases (11.5%) of the crushing injury group showed no clear growth. In contrast, samples from which grouping from laceration wounds had the highest non-growth rate, at 22 cases (30%), while the prevalence of *K. pneumoniae* was lower, at 14 cases (20%). The detection rate of pathogens other than *K. pneumoniae* was higher in crushing injuries (66.5%), while the rate of other pathogens in laceration wounds was (29.5%). Comparisons of *K. pneumoniae* isolation between the two groups of isolated wound samples revealed that crush injuries had a higher infection rate of 37% compared to lacerations, which had a higher infection rate of 20%. The results show a statistically significant association ($\chi^2 = 4.93$, $p = 0.026$), which confirms that crush injuries due to *K. pneumoniae* infection are more than twice as likely to be associated with lacerations compared to laceration wounds.

The results revealed that crushing injuries, which expose deep tissues to damage, ischemia, and necrosis, provide a favorable environment for colonization by *K. pneumoniae*, as well as other opportunistic pathogens. The results also showed that laceration wounds, despite their contamination from environmental sources, are relatively less susceptible to *K. pneumoniae* infection, perhaps due to the infection of superficial tissues rather than deeper tissues. Overall, 29.5% of these bacteria were isolated, reinforcing their key role in trauma-related wound infections, particularly those associated with deep and necrotic injuries. Demographic data at table 2 for patients with *K. pneumoniae* bacteria were collected due to crushing injuries and cut injuries that included sex, age, and type of wound and frequent use of antibiotics in addition to people with diabetes, the cause of the wound and the period of staying in the hospital. Demographic data from the study of 49 patients with wound infections caused by *K. pneumoniae* confirmed that males were 82% of cases, with a mean age of 37 years. Crushing trauma accounted for 71.4% of the cases, and lacerations accounted for (28.6%). The study also revealed that 44.9% of patients had a history of repeated antibiotic use, while (24.5%) of them had diabetes. Their hospital stay ranged from 2 to 13 days, with a mean of approximately 5.76 days. A statistically significant association was found between diabetes status and prior antibiotic use ($\chi^2=16.67$, $p < 0.0001$), showing that diabetic patients were more likely to have taken antibiotics prior to their accident and hospital admission. Also, no statistically significant association was seen between gender and the cause of injury ($\chi^2=0.0043$, $p=0.953$) or between diabetes and the cause of injury ($\chi^2=0.0047$, $p=0.495$). Antibiotic susceptibility profiles were analyzed for 49 patients, distinguishing between ESBL-producing and non-ESBL-producing isolates. The results showed that the non-producing strains had high sensitivity ($\geq 80\%$) to all types of antibiotics tested including cephalosporins (Cephalexin, Cefotaxime, Ceftazidime, Ceftriaxone), Cefepime, and Imipenem, while the ESBL producing strains showed clearly low sensitivity, especially to the third generation cephalosporin such as Ceftriaxone (0%), Ceftazidime (~13%), and Cefotaxime (~31%). There was also a decline in the effectiveness of cefepime, a fourth-generation cephalosporin antibiotic (21%) against bacteria with ESBL-producing strains. Age-stratified analysis also showed that the sensitivity of non-ESBL-producing strains remained high across all patient age groups, while the sensitivity of ESBL-producing strains was low across all age groups, with a small exception in the 30-50 age groups for some drugs such as Imipenem and Cephalexin. The strains producing ESBLs were considered highly resistant across all age groups of patients in the study, particularly to beta- lactams, highlighting the size of the clinical challenge they pose. These findings underscore the urgent need for prudent antibiotic stewardship and the development of tailored treatment strategies to address ESBL-producing bacteria. Phenotypic resistance to gentamicin was recorded in 39 (79.6%) of patients infected with *K. pneumoniae*, while 31 (63.3%) of samples were resistant to kanamycin. About molecular tests, *aac (3)-II* gene was found in 41(83.7%) samples, where all samples were resistant to gentamicin, while the samples of the other eight patients did not have this gene and were therefore also susceptible to infection. Likewise, the *aph (3) - Ia* gene was found in 33 samples (67.3%) of patients infected with *K. pneumoniae* bacteria, representing 67% of samples, where the samples were resistant to kanamycin, while the 16 samples of infected patients that did not have this gene were also susceptible to infection.

The Current study proves a statistically significant association between the presence *aac (3) - II* and resistance mechanisms of aminoglycoside antibiotics, and *aph (3') - Ia* a perfect one-to-one correlation between these genes' resistance. Isolates that evaluated positive for the *aac (3)-II* gene typically showed resistance to gentamicin and tobramycin, which aligns with the gene's known ability to change and inactivate these drugs. Likewise, those with the *aph (3) - Ia* gene were mostly resistant to kanamycin and neomycin, consistent with its role in antibiotic inactivation through phosphorylation. This combined approach using molecular and phenotypic methods allowed us to confirm that the presence of these genes correlates with actual drug resistance in the isolates.

Clinical samples from patients infected with *K. pneumoniae* carrying both genes showed resistance to both antibiotics, while the remaining ten samples were believed to have these genes through transfer mechanisms. There were three clinical samples of patients infected with *K. pneumoniae* that were individually resistant to gentamicin. This confirms the absence of any genotype-to-phenotype correlation between mismatches in our study and the significant role of these genes in aminoglycoside antibiotic resistance. Therefore, statistical analysis ($p > 0.0001$) shows a high significance.

Table (5): Wound infection outcomes by type

Wound Type	Total Specimens	Non-growth Samples	Positive of isolates <i>K. pneumoniae</i>	Other bacteria	Infection Risk
Crashing injury	95	11 (11.5%)	35 (37%)	63 (66.5%)	Deep tissue damage - Ischemia - Necrosis
Laceration Wound	71	22 (30%)	14 (20%)	21 (29.5%)	Open injury
Totally	166	33 (20%)	49 (29.5%)	84 (50.5%)	

Table (6): Age wise Antibiotic Susceptibility by ESBL Status

Age group	ESBL Producer	generations of cephalosporins					Carbapenems Imipenem
		First- Generation (Cephalexin)	Third - Generation			Fourth- Generation (Cefepime)	
			Cefotaxime	Ceftazidime	Ceftriaxone		
<30	ESBL- Kp	0.33	0.16	0.16	0.0	0.0	0.0
<30	Non- ESBL- Kp	1.0	1.0	1.0	1.0	1.0	1.0
30-50	ESBL- Kp	0.31	0.36	0.13	0.0	0.27	0.31
30-50	Non- ESBL- Kp	0.83	1.0	1.0	1.0	1.0	1.0
51-70	ESBL- Kp	0.2	0.2	0.1	0.0	0.2	0.2
51-70	Non- ESBL- Kp	0.5	1.0	0.5	0.5	1.0	1.0
70+	ESBL- Kp	1.0	1.0	0.0	0.0	0.0	0.0
70+	Non- ESBL- Kp						

Table (7): Resistance genes Patterns of Aminoglycoside in *K. pneumoniae* isolates

Genes present	Gentamicin Resistant	Kanamycin Resistant	<i>aph (3')-Ia</i> gene	<i>aac (3)-II</i> gene	Count isolates
None (-/-)	Susceptible (S)	Susceptible (S)	-	-	10
<i>aac (3)-II</i> gene only (+/-)	Resistant (R)	Susceptible (S)	-	+	3
Both genes present	Resistant (R)	Resistant (R)	+	+	36
Total	39 (R) / 10 S	31 (R) / 18 (S)	33(+)	41 (+)	49

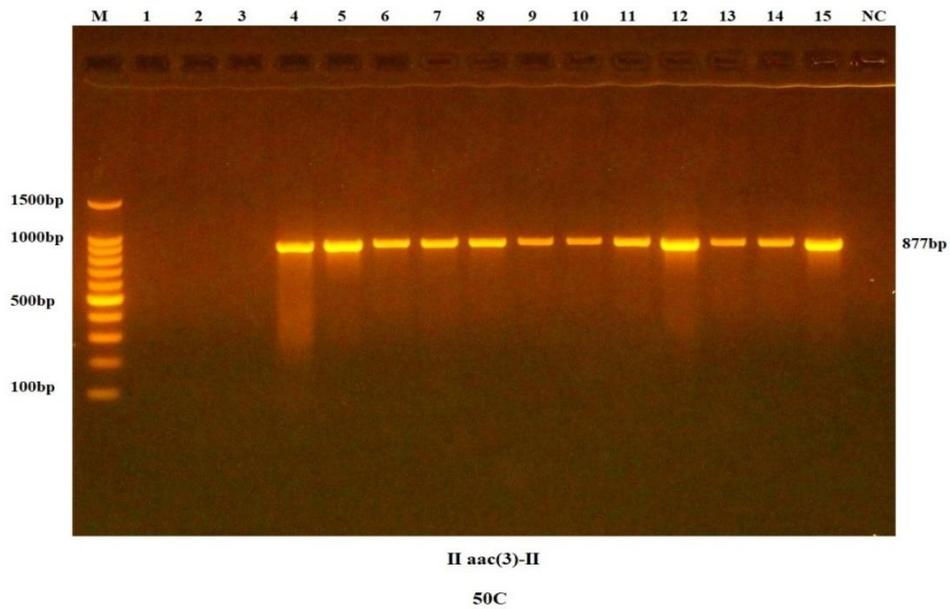


Figure (1): Amplification of II aac(3)-II gene of *Klebsiella pneumoniae* samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 877 bp PCR products, NC: negative control.

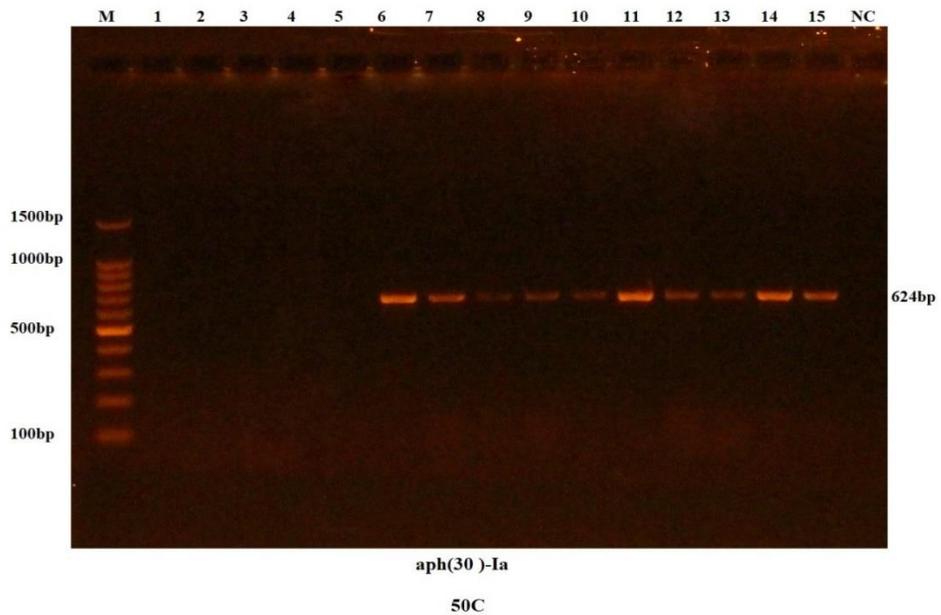


Figure (2): Amplification of aph(3)-Ia gene of *Klebsiella pneumoniae* samples species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-15 resemble 623 bp PCR products, NC: negative control

4- DISCUSSION

The difference in isolation rates between the two types of wounds (crushing injuries, laceration wounds) in the study suggests that deep tissue damage, ischemia, and necrosis provide an anaerobic and immunologically favorable environment for the colonization and multiplication of opportunistic Gram-negative bacteria such as *K. pneumoniae*, making bacterial survival and resistance to phagocytosis more likely than other conditions [14]. While, laceration wounds cases showed a lower prevalence of bacterial colonization, as they are exposed to air despite a greater risk of infection from environmental contaminants and skin bacteria [15]. This is also consistent with earlier studies showing that deep tissue infections accompanied by poor blood flow are more likely to be conducive to bacterial colonization; particularly Gram-negative ones. This study is consistent with published studies on ESBL-producing bacteria. Our data showed complete resistance to ceftriaxone, but extremely low sensitivity to ceftazidime (~13%) and cefotaxime (~31%) among ESBL-producing *K. pneumoniae* strains. This reflects the findings of [16, 17, 18].

Whose study reported widespread resistance to third-generation cephalosporins even in ESBL-producing bacteria? Imipenem, a carbapenem, showed high efficacy against ESBL-producing strains in the study population and is therefore the preferred treatment way, as shown of Merino trial [13]. Cefepime, a fourth-generation cephalosporin, has shown efficacy (~21%), but there are clinical studies [18], caution against its use due to its high failure rates as a treatment. There was no significant difference in resistance patterns between age groups in our study, consistent with who showed that resistance was more closely related to bacterial factors than to patient demographics in patients infected with *K. pneumoniae*. The data revealed by our study supports the increasing need for cautious antibiotic use, rapid detection of ESBL-producing strains, and empirical avoidance of cephalosporin antibiotics when ESBL infection is suspected [19, 20]. The results of Table 4 show a statistically significant association between aminoglycoside antibiotic resistance genes and resistance tests in clinical isolates. All isolates carrying the *aac (3) - II* gene (n=41) were resistant to gentamicin, while isolates without it (n=33) were completely susceptible. In contrast, all *K. pneumoniae* isolates carrying the *aph (3')-Ia* gene (n=33) were resistant to kanamycin, while other strains of the same bacteria that were not susceptible were susceptible.

The results in Table 4 are consistent with earlier studies describing *aac (3)-II* and *aph (3')-Ia* as reliable markers of gentamicin and kanamycin resistance, respectively [21, 22]. Another study reported similar correlations between genotype and phenotype by previous findings [23], further strengthening the predictive power of molecular diagnostics in improving overall antibiotic resistance management. Given the increasing incidence of antibiotic resistance worldwide, the current findings in our study highlight the importance of continuing to routinely integrate molecular surveillance of resistance genes into hospital and healthcare laboratories. Understanding the distribution of resistance determinants within clinical groups can help guide empirical treatment options and inform infection control policies [23, 24].

4- CONCLUSION

The study revealed widespread *K. pneumoniae* prevalence and significant resistance, with crushing injuries having a higher infection risk due to the nature of the trauma and tissue damage. The same bacteria, which produce ESBLs, showed significant resistance to cephalosporins and, in some cases, even to carbapenems in older age groups. Age and ESBL production are critical factors in determining appropriate antibiotic therapy, so empiric therapy should be considered in suspected ESBL infections. There was a strong association between the presence of the *aac (3)-II* gene and gentamicin resistance and the *aph (3')-Ia* gene and kanamycin resistance, with the presence of both genes in most of the study isolates accounting for the high level of dual resistance observed. Thus, the genetic data clearly explain the apparent resistance patterns. Recommendation: Early Aggressive wound management by debridement devitalized tissue using Low-pressure irrigation flushing a wound using gentle pressure; it does not damage the tissue and antiseptic decontamination. Empirical Antibiotic prophylaxis therapy for all crush and open injuries, an extended spectrum β -lactam to cover gram negative. When they are not available, we can use Colistin or tigecycline as salvage therapy. Narrow therapy is to the maximum effective, least toxic agent Limit duration of systemic antibiotics to the shortest effective course 5–7 days for uncomplicated soft-tissue infections.

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