Malaysian Journal of Microbiology, Vol 20(1) 2024, pp. 40-45
DOI: http://dx.doi.org/10.21161/mjm.230289

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Capsular specific typing of multidrug-resistant *Klebsiella pneumoniae* isolated from clinical cases in Ramadi city of Iraq

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Received 19 May 2023; Received in revised form 1 October 2023; Accepted 5 October 2023

ABSTRACT

Aims: The increasing incidence of *Klebsiella pneumoniae* infections in the community and hospitals is a considerable health problem. This is due to the rising resistance of the bacteria to antibiotics, biofilm formation and the presence of a capsule. The aim of this study was to survey the most common capsular types in local isolates for the first time in Iraq on a molecular level.
Methodology and results: Seventy isolates were screened for multidrug resistance (MDR) using a standard test. Genomic DNA was extracted from all isolates and PCR was performed using a multiplex PCR assay to detect the capsular type genes for K1, K2, K5, K20, K54 and K57. Forty-eight (68.5%) isolates demonstrated resistance to at least one agent of three or more antimicrobial categories and were therefore considered as MDR isolates. Multiplex PCR showed that 16/48 (33.3%) of MDR isolates belonged to the K2 capsular type and two isolates belonged to the K57 capsular type. The other four capsular types were not detected.
Conclusion, significance and impact of study: The K2 capsular type was the most common capsular type among MDR *K. pneumoniae* isolates from urinary tract infections (UTI) in Ramadi, Iraq. Monitoring capsular type is essential in addition to monitoring antibiotic resistance, as highly resistant strains with hypervirulent types can be particularly dangerous.

Keywords: Capsule, Iraq, *Klebsiella pneumoniae*, K2, MDR

INTRODUCTION

Klebsiella pneumoniae is an important human pathogen in both hospital and community settings, which causes nosocomial infections, such as septicemia, pneumonia, urinary tract infection, surgical site infections and catheter-related infections are associated with community-acquired infections (Lin *et al.*, 2010).

The virulence of *K. pneumoniae* is determined by many factors, including the polysaccharide capsule that allows to evade phagocytosis process by the host, type 1 and 3 fimbriae responsible for bacterial cell adhesion to host surface (Paczosa and Mecsas, 2016) and various siderophores used for uptake of iron (Miethke and Marahiel, 2007). *Klebsiella pneumoniae* is classified into classical *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae (hvKp)* (Zhu *et al.*, 2021). Several hyper-virulent types of *K. pneumoniae* have genes associated with life-threatening invasive diseases.

Capsule is considered the main virulence factor of *K. pneumoniae* and capsular types are related to the severity of infection; the spread of capsular types in each *K. pneumoniae*-related disease could be critical for disease control and prevention (Cortés *et al.*, 2002).

Klebsiella pneumoniae produces an acidic capsule polysaccharide (CPS), which is considered an important factor for survival in the hosts. Based on the diversity of the polysaccharide components of the capsule and different structures and antigens, *K. pneumoniae* can be divided into at least 79 serotypes (Zhu *et al.*, 2021). The capsule polysaccharides (CPS) are composed of many sugar units, which make a protective layer of material on the bacterial surface. Multiple genes that comprise the CPS locus are involved in capsule biosynthesis. The assembly and translocation of CPS onto the bacterial surface are regulated by proteins encoded by the conserved genes *galf or f2*, *wzb*, *wza*, *wzi* and *wzc* at the 5' end of the cps locus. The variation in the nucleotide sequences and gene numbers underlies the differences in *K. pneumonia*e capsular type (Shu *et al.*, 2009).

The *wzi* and *wzy* genes are found in each capsule type, but each has a high rate of variable sequence

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 40-45
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between specific capsule types (Turton *et al.*, 2010; Fevre *et al.*, 2011). Mostly, the most virulent strains of *K. pneumoniae* have produced a capsular make of saccharide that does not encourage binding to phagocytic cells, thereby more resistance to phagocytosis. This study focused on multidrug-resistance resistant *K. pneumoniae* isolates and aimed for the first time to detect the common capsular type in Ramadi city teaching hospitals of Iraq on a molecular level.

MATERIALS AND METHODS

Bacterial sampling

This study was conducted at the Biotechnology Department of Anbar University in the period from March 2022 to April 2023. Bacterial samples were collected from clinical settings, including patients of both sexes and all ages who suffered from UTIs and pneumonia. Additionally, samples were collected from surgical site infections and catheter-related infections from different teaching hospitals in Ramadi/Iraq.

Cultivation of suspected microbial samples

All samples were cultivated on MacConky and blood agar using the streaking plate method under antiseptic conditions; then, these samples were incubated at 37 °C for 18 h.

Laboratory identification

The samples identified by traditional microbiological methods, which include colonial morphology, Gram staining, Biochemical tests and the Vitek 2 Compact system, was utilized for the final identification of the organism using a GN (Gram-negative) identification card, as per the manufacturer's instructions (bioMérieux, France).

Antibiotics susceptibility test

For each pure isolate, the antimicrobial susceptibility test was done according to the Kirby-Bauer disc diffusion method as described in the guidelines of the CLSI Institute (CLSI, 2021) and using an AST card by automated Vitek-2 compact system.

Some phenotypic confirmation tests for extended-spectrum β-lactamases (ESBLs) production

Modified Hodge test (MHT)

In order to identify ESBLs and carbapenems producing *K. pneumoniae*, all β-lactam-resistant strains susceptible isolates were identified and then examined by phenotypic (MHT) according to (Ahmed and Al Meani, 2019). Clover-leaf result gave an indicator for positive for this test.

Double disk synergy test (DDST)

An amoxicillin-clavulanate disk was placed at 20 mm to the center of cefotaxime ceftazidime, cefpodoxime, aztreonam or cefepime disks on a Mueller-Hinton agar plate and incubated at 37 °C for (18-24 h) and the remaining steps were done according to (EUCAST, 2019; Fazal, 2019).

Modified cephalosporine inactivation methods (mCIM) and eCIM

It is used for detecting cephalosporinase in Enterobacteriaceae. On the other hand, eCIM was used to differentiate metallo-β-lactamases from serine cephalosporinase in Enterobacteriaceae after adding EDTA. These methods were done according to (CLSI, 2021).

String test

Differentiation between classical *K. pneumoniae (*cKp*)* and hypervirulent *K. pneumoniae* (hvKp) using string test according to Vuotto *et al.* (2017).

Extraction of bacterial genomic DNA

The bacterial DNA (deoxyribonucleic acid) was extracted according to the Genomic DNA mini-Kit provided by

Table 1: Sequence of capsule-type primers and their gene size.

Gene	F/R	Sequence of primer 5' - 3'	PCR product size bp	Reference
Capsular type K1	F	GGTGCTCTTTACATCATTGC	1283	
	R	GCAATGGCCATTTGCGTTAG		
Capsular type K2	F	GACCCGATATTCATACTTGACAGAG	641	
	R	CCTGAAGTAAAATCGTAAATAGATGGC		
Capsular type K5	F	TGGTAGTGATGCTCGCGA	280	
	R	CCTGAACCCACCCCAATC		(Turton *et al.*,
Capsular type K20	F	CGGTGCTACAGTGCATCATT	741	2010)
	R	GTTATACGATGCTCAGTCGC		
Capsular type K54	F	CATTAGCTCAGTGGTTGGCT	881	
	R	GCTTGACAAACACCATAGCAG		
Capsular type K57	F	CTCAGGGCTAGAAGTGTCAT	1037	
	R	CACTAACCCAGAAAGTCGAG		

F, Forward primer; R, Reverse primer.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Geneaid Company.

DNA quality evaluation

A Nano-drop device examined the extracted DNA purity and concentration.

Agarose gel electrophoresis

Agarose gel electrophoresis was adopted when genomic DNA extraction was done to confirm the presence and integrity of DNA. These methods were done according to Boffey (1984).

Polymerase chain reaction (PCR)

All PCR reactions were done in Applied Bio-system 2720 thermo cyclers. Multiplex PCR was carried out using primers for six gene targets; the details are listed in Table 1. Reactions were carried out according to Turton *et al.* (2010). Most PCR reactions of *Escherichia coli* 25922 genomic DNA were used as a negative control.

For the PCR program, the initial denaturation step for each PCR assay with different primers was started at 95 °C for 4 min. In subsequent cycles, the denaturation was done at 94 °C for 30 sec. The annealing time was 50 sec for all primers and temp at 58 °C. The extension time was 1 min at 72 °C. The final extension for all genes was done at 72 °C for 7 min. On the other hand, the reaction of PCR consisted of 12.5 µL PCR Master mix, 0.5 µL forward and reverse primer, 5.5 µL Free-nuclease water and 1 µL DNA template. The final volume was adjusted to 25 µL.

Statistical analysis

All statistical analyses were performed using SPSS, version 18.0 (SPSS Inc., NY, USA). Chi-square tests were used to compare the relationship of capsule type with the string test.

Ethical approval

The study was approved by the Ethics Committee of the Al Anbar Medical Research University (approval number 205, December 29, 2022). All individuals/subjects had given consent to participate in the current study. In addition, subjects under 16 years old were not included in the study.

RESULTS AND DISCUSSION

Sampling and isolation

A total of 345 specimens were collected from clinics. Samples were from both sexes of different ages, who suffered from pneumonia infection, UTI and surgical site infections. Samples were also collected from catheter-related infections from Al-Ramadi Teaching General Hospital and Al-Ramadi Teaching for Children and Maternity Hospital.

Out of 345 specimens, 258 (74.7%) were positive for culturing, while 87 (25.3%) were negative for culture. The identification of isolates according to colony morphology (Figure 1), biochemical tests (Table 2) and confirmed by Vitek 2 compact device as a final identification of organisms by using a GN (Gram-negative) card, which showed that 70 isolates belong to *K. pneumoniae*. Urine samples were the most frequent for *K. pneumonia*e identification [n=45 (64.2%)], followed by sputum [n=15 (21.4%)], blood [n=5 (7.1%)] and catheter-related infections [n=5 (7.1%)].

The negative culture cases were attributed to the reasons that some patients were under antimicrobial chemotherapy during culture time (Al-Ouqaili *et al.*, 2018).

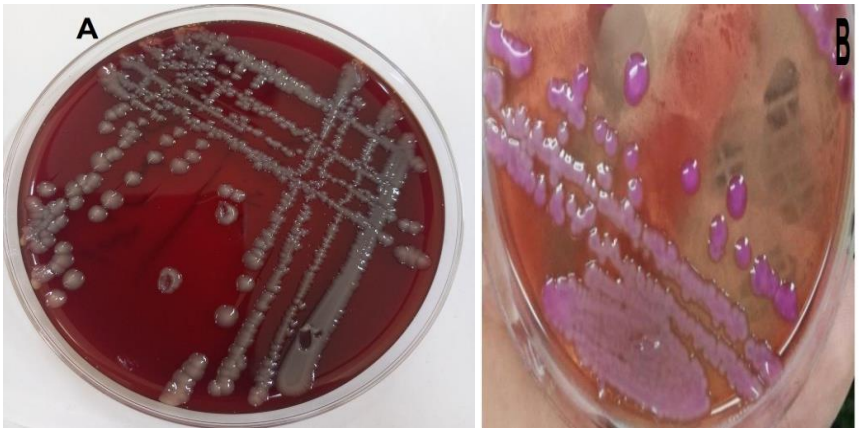

Figure 1: A: *K. pneumoniae* on blood agar; B: *K. pneumoniae* mucoid, large, pink, lactose fermenter colonies on macConkay.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Table 2: Biochemical tests for *K. pneumoniae*.

Test	*K. pneumoniae*
MacConkey agar medium	Lactose ferment (+)
Gram-stain	G-rod
Catalase test	+
Oxidase test	-
Indole test	-
Methyl red test	-
VP test	+
Citrate utilization test	+
Urease	+

Antimicrobial susceptibility pattern

As determined by Kirby Bauer disks diffusion method according to the recommendation of CLSI (CLSI, 2021), and using AST card by automated Vitek-2 compact system.

All isolates (70) tested for seven antimicrobial agents, including Augmentin, Ceftazidime, Ceftriaxone, Amikacin, Ciprofloxacin, Meropenem and Cefotaxim. The susceptibility results of the screening for MDR isolates showed that there was a high level of diversity in antibiotic resistance among the *K. pneumoniae* isolates when the resistance to at least one agent of three or more antimicrobial categories were detected in more than 68.5% (48/70) of the isolates and considered as MDR isolates.

The local study conducted in Ramadi city of Iraq showed that *K. pneumoniae* possesses the highest resistance to third-generation of cephalosporins and that agreement with many local studies, resulting from the widespread distribution of ESBL-producing. On the other hand, the plasmid has a vital role in acquiring and transmitting resistant determinants option and virulent genes in *K. pneumoniae* (Salim *et al.*, 2017).

The percentage of carbapenem resistance has increased. In contrast, one of the most prominent recent increases in MDR was observed with *Klebsiella* spp. in the period from 2001 through 2018 (Wasfi *et al.*, 2016), in which carbapenems were considered as a last line drug to the treatment of infections caused by multidrug-resistant *K. pneumoniae*. However, it is noteworthy that patients with infections due to carbapenemase-producing *K. pneumoniae* experience high mortality rates.

The world fears that MDR or extensively drug-resistant (XDR) isolates may turn into full resistance toward antibiotics SUPERBUGS BACTERIA as a result of superior resistance to antibiotics in the treatment of bacterial infections, which is called pan drug-resistant (PDR). So, the results of this study found that five isolates are XDR, and three isolates are PDR. These percentages vary from one city to another within the country, as reported by (Ahmed and Al Meani, 2019). Of all the MDR isolates surveyed, two were found to be sensitive to all antibiotics. This is a promising finding, suggesting that some individuals are still following unique health systems promoting antibiotic stewardship. The spread of PDR bacteria is a significant threat to global health, and it is essential to take steps to prevent the development of these strains; they are difficult or impossible to treat with traditional antibiotics and can cause serious infections that are difficult to cure.

Some phenotypic confirmation tests for extended spectrum β-lactamases production

Thirty isolates were selected for the screening of ESBL production, which were resistant to the third generation of cephalosporins and have given a positive result by containing the extended-spectrum β-lactamase as follows:

Modified Hodge test (MHT)

The study results showed that 10/30(33.3%) gave a positive result for MHT; this test is considered a simple that can be performed in the routine lab for the detection of β-lactamases in isolates that show intermediate or sensitive zone diameter in disc diffusion (Amjad *et al.*, 2011).

Modified cephalosporins inactivation method (MCIM)

The result of MCIM for ESBLS production among cephalosporin-resistant *K. pneumoniae*, showed that all isolates a gave positive result that agreed with (Ahmed and Al Meani, 2019)

Double disk synergy test (DDST)

The result shows that 20/30 (66.6%) isolates were positive for DDST. Disks of Augmentin and third-generation Cephalosporin have been kept at 20 mm apart, center to center, on the Mueller-Hinton agar. The result from the extension of the inhibition zone region of Cephalosporin to the Augmentin disk is considered a positive for extended spectrum β-lactamase production. Evaluations of the DDST have revealed specificities ranging from 94% to 100% and the sensitivity of this method ranging from 79% to 97% (Rawat and Nair, 2010).

ESBLs are a group of plasmid-mediated, diverse, complex, and rapidly evolving enzymes that pose a significant therapeutic challenge today in the treatment of hospitalized and community-based patients. Infections due to ESBL producers range from uncomplicated UTIs to life-threatening sepsis. β-lactamases, which enzymes have the ability to hydrolyze third-generation cephalosporins and aztreonam. In general, the organisms that produce these enzymes are resistant to other classes of antibiotics and, thus, limit therapeutic options. The general principle in the detecting methods of ESBL depends on enhancing the activity of spectrum cephalosporins against ESBL-producing organisms through a clavulanate substance. Carbapenems are the best choice for many infections caused by ESBL-

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 40-45
DOI: http://dx.doi.org/10.21161/mjm.230289

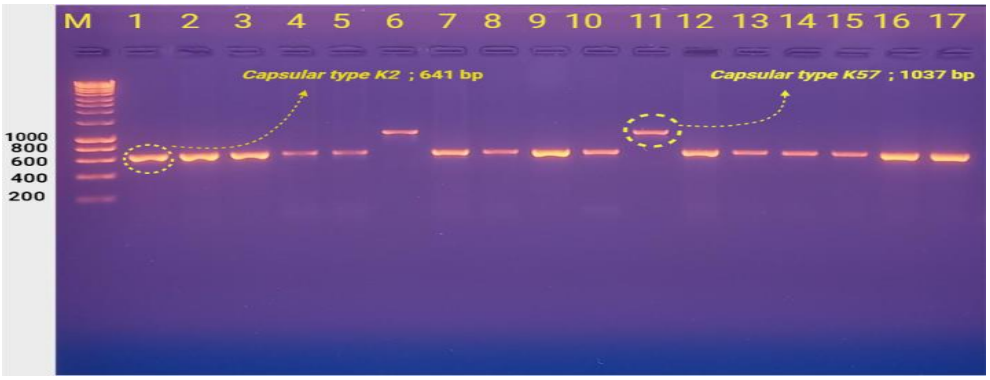

Figure 2: Multiplex-PCR amplification fragments for detecting capsular type (1.5% agarose, 7 V/cm^2 for 90 min). Lanes 6 and 11 are *K. pneumoniae* capsule types *K57*; 1-17: *K. pneumoniae* capsule type *K2*; Lane M: 200-bp DNA ladder.

producing bacteria, but recently, many cases resistant to these antibiotics have been recorded (Rawat and Nair, 2010), as the current study found.

Molecular screening of capsules

All MDR isolates were subjected to genomic extraction and PCR analysis. The results of multiplex PCR electrophoresis (Figure 2) showed that capsular K2 type was the most common in local isolates, accounting for 33% (16/48) and 2 isolates belonged to the K57 capsule type. This result is consistent with a previous study by (Feizabadi *et al.*, 2013). The study results indicate that K2 capsule type is most associated with invasive disease or pathogenicity in local patients, where it is often a nosocomial infection, and this may explain the bacteria's resistance to antimicrobial agents. The other common capsule types (K1, K5, K20 and K54) were not detectable by PCR, which may be interpreted as these capsule types are not common in local isolates.

The study results were inconsistent with the global known foundation about the distribution of capsular type. Because the prevalent serotypes differ with the geographic regions which the K2 capsular type is more frequently found in Europe and North America while K1 is the most predominant type in Asia (Remya *et al.*, 2018), perhaps the transfer of these isolates to Asian countries, such as Iraq, may be explained by the increase in tourism or commercial activity between the continent of Asia and Europe, which led to the rise of K2 capsule type frequency.

The survey and determination of capsular types is considered a global world target in futurity to treatment of *K. pneumoniae* infection by capsule as a target for therapeutics and vaccines due to difficulty to treatment because these microbes are usually resistant to many antimicrobial agents.

Association of capsule with MDR - Hypervirulent strains

The results showed a statistically significant relationship between the presence of capsule and string test at P-value (0.0324), so most of the isolates that were positive for string test have a capsular type (K2). All isolates that are positive to PCR amplification for capsule type demonstrate a high resistance toward antibiotics. This may be interpreted as the failure of the drug to arrive at its own target in cells because the capsule (K antigen) is considered the first layer in a confrontation of drugs, so the isolates that were considered hyper-mucoviscosity (hypervirulent strains) were more resistance. On the other hand, the results of some hypervirulent strains demonstrate that although these strains are hypermucoviscous but were sensitive to carbapenems antibiotics, that may back to the thick hyper-capsule of hvKp strains can act as a physical barrier that prevents the uptake of DNA. This limits the horizontal gene transfer of antimicrobial-resistant genes, which may be one reason why hvKp strains are less likely to harbor these genes than cKp strains. (Wyres *et al.*, 2019). So, the growing coexistence of these conditions is of particular apprehension as it can lead to untreatable and invasive *K. pneumonia* infection.

The presence of the capsule polysaccharide around the microbial cells protects them from the process of phagocytosis by masking the O antigens and thus hindering the detection of the microbe by the phagocytes (Hasani *et al.*, 2020). Therefore, the presence of the capsule is the most important factor for bacterial virulence by preventing the process of phagocytosis, so, the strains that have a special type of capsule are considered more virulent strains and evade the immune system (Opoku-Temeng *et al.*, 2019).

CONCLUSION

Based on the results and its analysis, it is concluded that capsule K2 type had been a high frequency among MDR *K. pneumoniae* isolated from urinary tract infection with relation to the capsule's presence with hypervirulent strains. Careful monitoring at the level of transferable determinants is required to ensure that highly resistant strains do not spread. Further research is required.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538