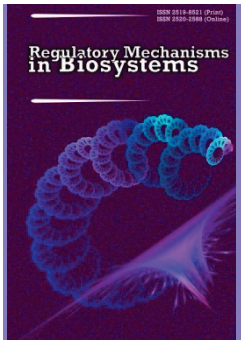

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Molecular identification of some virulence genes in *Klebsiella pneumoniae* isolated from different clinical cases

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The aim of this work was to acquire a better understanding of the molecular epidemiological aspects of *Klebsiella pneumoniae* in Iraq, which is critical for the prevention and management of *K. pneumoniae* infection and transmission. Two genes involved in antibiotic resistance, bla_{TEM} and bla_{SHV}, were detected. Results showed that 100% of the isolates exhibited both bla_{TEM} and bla_{SHV}, which suggests that both genes are carried on the same plasmid due to the fact that they were presented in all isolates. The presence of *wzi*, a gene required for capsular polysaccharide, and *rmpA*, a capsule synthesis accelerator, was detected in all *Klebsiella* isolates, indicating the importance of both genes in antibiotic-resistant *K. pneumoniae* and demonstrating that all *Klebsiella* strains under study are polysaccharide producers and thus may be strong biofilm producers. This study reveals that the clinical isolates randomly selected were highly pathogenic which is considered a threat to health providers in Iraq.

Keywords: *Klebsiella pneumoniae*; antibiotic resistance; biofilm formation; virulence genes.

Introduction

Klebsiella species are non-motile, gram-negative bacilli that are capsule-shaped and members of the Enterobacteriaceae family. A commensal bacterium called *Klebsiella pneumoniae* is present on healthy people's skin, in the gastrointestinal and respiratory systems (Paterson, 2006). It is also widely present in the environment. It is an opportunistic pathogen that can cause a variety of nosocomial and community-acquired diseases, including respiratory tract infections, wound and soft tissue infections, and urinary tract infections (UTIs) (Jasim et al., 2020). With a rising death rate, especially in the old, immunocompromised, and newborns, it has emerged as one of the global leaders in nosocomial infections in recent years. Additionally, it is becoming more and more linked to serious community-acquired illnesses including meningitis and pneumonia (Shah et al., 2010).

Antibiotic resistance is caused by both overuse and misuse of antibiotics, which raises serious concerns for public health. Because *K. pneumoniae* expresses virulence factors and develops antibiotic resistance, it is regarded as a pathogenic bacterium that underlies nosocomial illnesses. Antimicrobial resistance is becoming an increasingly serious problem for healthcare facilities globally (Joseph et al., 2021). Owing to the decreased efficacy of available treatments, pathogenic bacteria such as *K. pneumoniae* are rapidly evolving into multidrug-resistant (MDR) strains, which frequently represent a significant risk to patients due to a higher death rate (Dong et al., 2022).

To identify bacteria causing infections and determine antibiotic susceptibility, clinical bacteriology laboratories have historically depended on cultural characteristics, biochemical tests, and phenotypic approaches (Török et al., 2012). Traditional morphological and biochemical methods of bacterial identification have been gradually replaced by the 16S rRNA-based method, which has been widely acknowledged as a superior method since its discovery (Abayasekara et al., 2017). It is possible to identify known or new bacteria in uncommon infectious foci by using DNA sequencing of 16S rRNA genes, as this technique has occasionally led to the identification of entirely unexpected pathogens (Barghouthi, 2011). There is now a serious risk to the public's health because of the rise in inpatient mortality and medical expenses. The immune system uses a range of virulence factors, including capsular polysaccharide (CPS), lipopolysaccharide (LPS), fimbriae, iron acquisition, and biofilm, to help *K. pneumoniae* infections survive and escape (Russo et al., 2018). Variations in the virulence factors of *K. pneumoniae* result in variations in the pathogenic and clinical features. The purpose of this study was to gain a deeper understanding of the molecular epidemiological features of *K. pneumoniae* in this area, which is crucial for the prevention and management of *K. pneumoniae* infection and transmission (Remya et al., 2019).

Materials and methods

Sample collection. A total of 186 samples were collected from various pathogenic samples during the period from June to September 2023 from patients visiting Ibn Sina Teaching Hospital, and Mosul general Hospital Mosul city – Iraq.

Isolation and identification. Samples were cultured on blood agar and MacConkey agar plates and incubated for 24–48 hours at 37 °C. The initial diagnosis of the samples was made using the phenotypic, morphological, and cultivar characteristics of the CSF on the media. A chromium dye was used to stain pure cultures to observe the morphology of the cells and how they interacted with the dye. When necessary, basic biochemical assays including catalase and oxidase tests were carried out (Vandepitte et al., 2003).

API 20E and VITEK-2 system. Following the manufacturer's instructions, API 20E strips (BioMeurix, France) were used to diagnose a few Gram-negative bacteria to further corroborate the diagnosis. VITEK-2 system was used to further identify the probable strains.

Molecular diagnosis by 16S rRNA sequencing. Genomic DNA was extracted directly from a single colony from MacConkey agar using a genomic DNA isolation kit supplied by the Geneaid company. Steps were followed as recommended by the manufacturer. PCR was conducted using GoTaq G2 Green Master Mix supplied by Promega (USA), which was used in a 20 μL PCR volume. The 1495bp fragment from the 16S rRNA gene was amplified using the universal primers 27F: AGAGTT

TGATCMTGGCTCAG and 1522R: AAGGAGGTGATCCARCC GCA (Khaleel et al., 2023). The manufacturer's specified primer concentration and total template DNA quantity were applied. The initial denaturation of the 16S rRNA gene was set for 3 minutes at 95 ºC. This was followed by 30 cycles of amplification, which included denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 1 minute. The 72 ºC final extension step was established. After being separated on a 2% agarose gel, and stained with Intron Green Advance DNA stain. A 100bp DNA marker (Transgene China Company) was used as a molecular weight marker (Abdulrazzaq & Faisal, 2022).

The 16S rRNA gene PCR products were purified and delivered to the Psomagene sequencing company (USA) for sequencing. The NCBI's BLAST tool was used to scan the recovered sequences for homology against published genes that had been submitted to GenBank.

Analysis of biofilm-forming capabilities. The ability to create biofilms was assessed through the assessment of adherence to 96-well microtiter plates with a flat bottom. To put it briefly, 200 µL of sterile broth liquid medium was added to each well of the 96-well micro titration plates. Each well was filled with bacterial cultures that were in the biofilm formation stage (18 hours of incubation; 1:100 in liquid medium, 200 µL liquid medium). The only negative control that was employed was a liquid medium. Total cell mass was determined as absorbance at 570 nm (OD1) following a 48-hour incubation period at 37 ºC; the blank was measured at OD10. After three PBS washes, each well was dried for an hour and stained for 20 minutes using 200 µL of 1% crystal violet. After removing the crystal violet solution, each well was rinsed four times with PBS to eliminate any remained stains. After air drying for 30 minutes on the aseptic processing surface, 200 µL of 95% ethanol was applied to each well. After 30 minutes of vibration, absorbance at 570 nm (OD_2) was measured, with OD_{20} as the blank. The biofilm-forming capacity was determined using $B = (OD_2–OD_{20})/(OD_1–OD_{10})$. Strains were classified as no biofilm producers ($B < 0.1$), weak biofilm producers ($B \geq 0.1$), moderate biofilm producers ($0.1 < B \leq 1.0$), or strong biofilm producers ($B > 1.0$) based on their adhesion capabilities (Donlan & Costerton 2020).

Amplification of antimicrobial resistance genes. Two genes involved in ESBLs (bla_{TEM}, bla_{SHV}), and four genes involved in biofilm formation (*wzi*: encoding capsular polysaccharide, *fimH*: adhesion, *wabG*: lipopolysaccharide, and *rmpA*: mucous phenotype-related genes) were detected via conventional PCR in *K. pneumoniae* isolates. The primer information of these resistance genes is shown in Table 1 that displays PCR primer information for the pathogenicity genes under study (Hao et al., 2022).

Table 1
Gene names and primer sequences

Gene name	Primer sequence	Product size, bp	TM, ℃	Ref.
bla_{TEM}	P1: TCGCCGCATACACTATTCTCAG P2: ACGCTCACCGGCTCCAGATTTAT	445	55.1	30
bla_{SHV}	P1: ATGCGTTATATTCGCCTGTG P2: TGCTTTGTTATTCGGGCCAA	753	58	30
wzi	P1: GTGCCGCGAGCGCTTTCTATCTTGGTATTCC P2: GAGAGCCACTGGTTCCAGAA[CorT]TT[CorG]ACCGC	580	55	21
rmpA	P1 : ACTGGGCTACCTCTGCTTCA P2 : CTTGCATGAGCCATCTTTCA	516	58	34
wabG	P1 : ACCATCGGCCATTTGATAGA P2 : CGGACTGGCAGATCCATATC	683	49	34
fimH	P1 : TGCTGCTGGGCTGGTCGATG P2 : GGGAGGGTGACGGTGACATC	688	49	34

Results

Results showed that all *K. pneumoniae* isolates were biofilm producers. *Klebsiella pneumoniae* is recognized for its propensity to create biofilms, which are bacterial populations embedded in an extracellular matrix. This matrix contains proteins, exopolysaccharides, DNA, and lipopeptides. The presence of several genes involved in antibiotic resistance were detected. Two genes for ESBLs were studied, bla_{TEM} and bla_{SHV}, results showed that 11/11 (100%) of the isolates exhibited both bla_{TEM} and bla_{SHV} (Fig. 1 and 2).

The presence of the *wzy* gene essential for capsular polysaccharide was detected in all *Klebsiella* isolates (Fig. 3). *RmpA* was proposed as a pathogenic component alongside *magA* and *cps K1/K2*. Although *rmpA* is not an independent factor contributing to pyogenic liver abscess, it stimulates capsule synthesis, which is related to hypermucoviscosity. Our results showed that *rmpA* was present in 100% of the isolates, indicating that our isolates are strong biofilm producers and that the clinical isolates we randomly collected are strongly virulent.

Discusion

It has been found that antibiotic resistance in *K. pneumoniae* isolated from clinical specimens has grown year after year. However, research on molecular characteristics is still extremely rare. In the current investigation, 11 strains of *K. pneumoniae* were collected from Ibn Sina Hospital, Mosul-Iraq. The ability of *K. pneumoniae* isolates to form biofilm was studied. Results showed that all *K. pneumoniae* isolates were biofilm producers. The biofilm-forming phenomena and *K. pneumoniae* were initially described by LeChevallier et al. (1988). *Klebsiella pneumoniae* exploited these virulence factors for survival, evasion of the immune system during infection, and biofilm formation itself (Nirwati et al., 2019). ESBLs genes particularly bla_{TEM} and bla_{CTX} have been shown to be carried on plasmids (Schultsz & Geerlings, 2012). Our results suggest that both ESBLs genes are carried on the same plasmid due to the fact that they were present in all 11 isolates. Detection of ESBLs in 100% of the isolates raises many questions, as this is a very high percentage compared to other studies. Hae et al. (2022) showed that only 40% of *K. pneumoniae* isolated from sputum acquired bla_{TEM}. While Ahmad Hamad & Khadija (2019) showed that 64.7% of *Klebsiella* isolates acquired bla_{TEM} and 35.2% acquired bla_{SHV}.

The presence of the *wzy* gene essential for capsular polysaccharide was detected in all *Klebsiella* isolates, indicating the importance of this gene in antibiotic resistant *K. pneumoniae* and showing that all the studied *Klebsiella* strains are polysaccharide producers and therefore may be strong biofilm producers (Jasim & Farhan, 2020). This gene is an important candidate in molecular capsule typing (Hao et al., 2020).

Our results showed that *rmpA* was present in 100% of the isolates, indicating that our isolates are strong biofilm producers and that the clinical isolates we randomly collected are strongly virulent. Lower percentages of *rmpA* presence was detected in other studies. Liu et al. (2020) showed that (43.5%) of the 202 *K. pneumoniae* isolates carried *rmpA*. The presence of this gene was shown to be linked to hyper mucoid strains while it was not found in non-hyper mucoid strains (Wiskur et al., 2008).

wabG and *fimH* were not detected in all studied *K. pneumoniae* isolates, which might refer to the variation of our local strains in such genes which restricted the corresponding primers' ability to bind and amplify the genes (Abd Alrazzaq & Faisal, 2022). The emergence of virulent *K. pneumoniae* in clinical isolates in Iraq indicates a need for improved clinical awareness and infection monitoring.

Fig. 1. Amplicons of blaTEM (445 bp) amplified from *Klebsiella* isolates

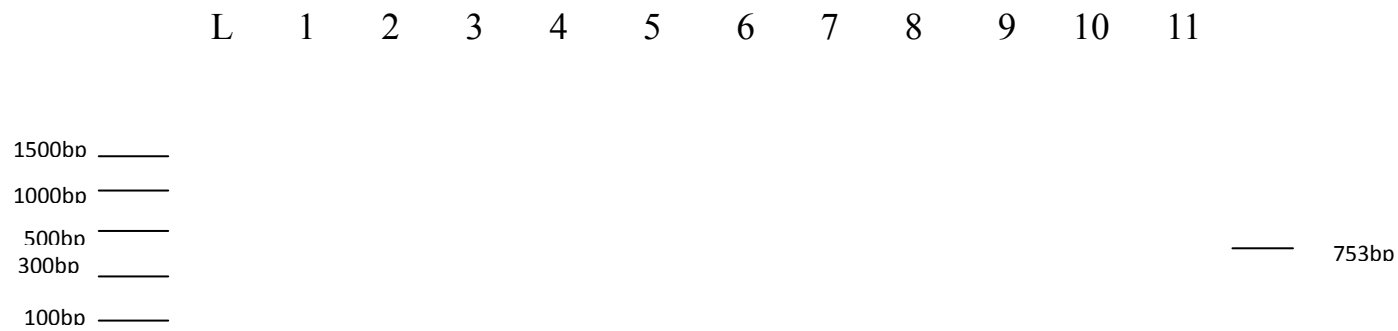

Fig. 2. Amplicons of blaSHV (753 bp) amplified from *Klebsiella* isolates

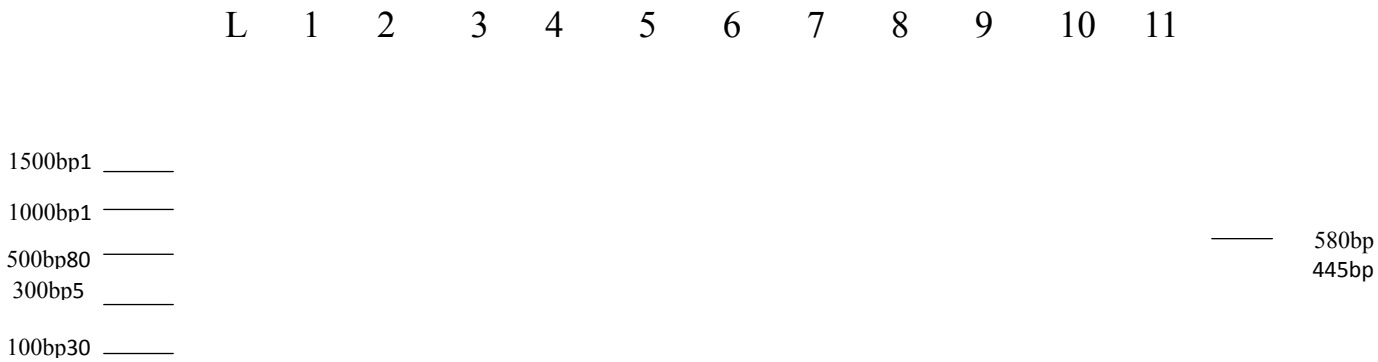

Fig. 3. Amplicons of wzi (580 bp) amplified from *Klebsiella* isolates

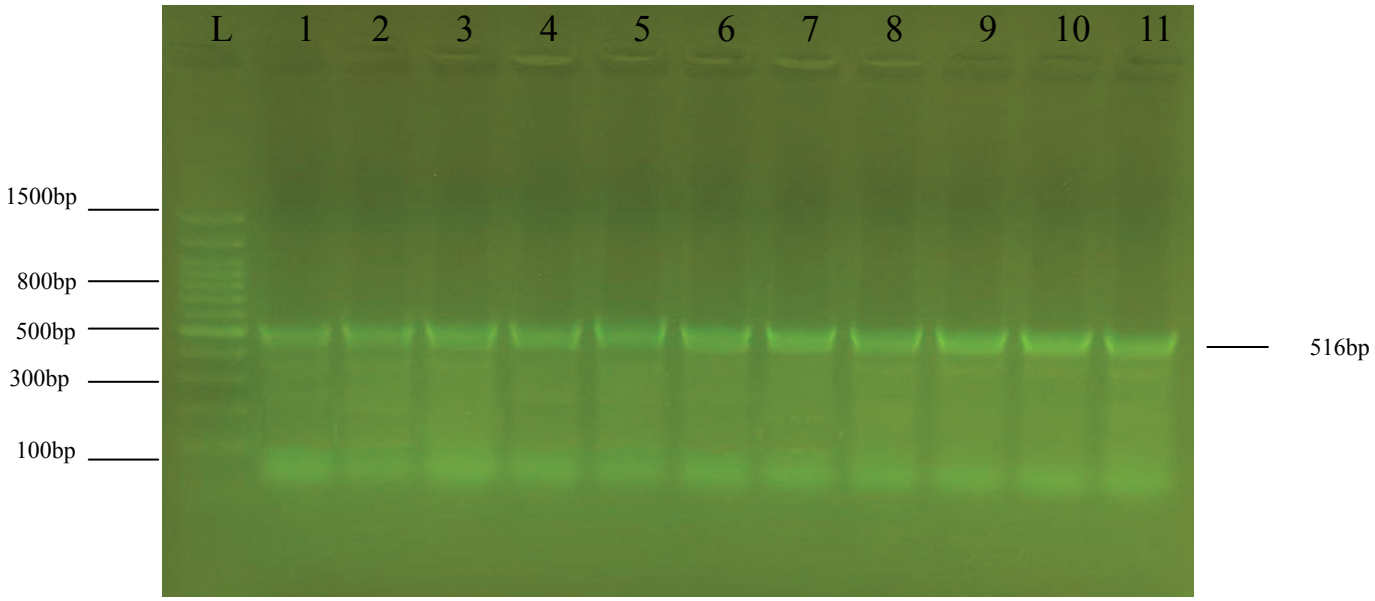

Fig. 4. Amplicons of rmpA (516 bp) amplified from *Klebsiella* isolates

Conclusion

This study found a high prevalence of virulence gens and antibiotic resistance genes in *K. pneumoniae*, which create biofilms, among Iraqi patients. In the current study, all isolates formed robust and moderate biofilms.

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