Original Article

Molecular characterization of the whole genome in clinical multidrug-resistant strains of *Klebsiella pneumoniae*

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Abstract
Introduction: Antimicrobial resistance (AMR) is a major public health concern. The spread of AMR-encoding genes between enterobacteria, especially in *Klebsiella pneumoniae* strains, lead to failure in the treatment of most individuals. The aim of this study was to characterize multi-drug resistant (MDR) clinical *K. pneumoniae* isolates that produce extended-spectrum β-lactamases (ESBLs) from Algeria.

Methodology: The isolates were identified using biochemical tests, and the identification was confirmed by mass spectrometry using VITEK® MS (BioMerieux, Marcy l’Etoile, France). Antibiotic susceptibility testing was assessed by the disk diffusion method. Molecular characterization was performed by whole genome sequencing (WGS) using Illumina technology. Sequenced raw reads were processed using bioinformatics parameters: FastQC, ARIBA, and Shovill -Spades. Multilocus sequence typing (MLST) was used to estimate the evolutionary relationship between isolate strains.

Results: Molecular analysis resulted in the first detection of blaNDM-5 encoding *K. pneumoniae* in Algeria. Other resistance genes were blaTEM, blaSHV, blaCTX-M, aac(6’)-Ib-cr, qnrB1, qnrB4, qnrB19, qnrS1, gyrA and parC variants.

Conclusions: Our data demonstrated a very high level of resistance in clinical *K. pneumoniae* strains which were resistant to most common antibiotic families. This was the first detection of *K. pneumoniae* with the blaNDM-5 gene in Algeria. Surveillance of antibiotic use and measures for control should be implemented to reduce occurrence of AMR in clinical bacteria.

Key words: antimicrobial resistance, extended-spectrum β-lactamases, *Klebsiella pneumoniae*, multi-drug resistant, New Delhi metallo-β-lactamases -5, sequenceType -307.


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Introduction

Prolonged exposure of Enterobacteriaceae to antibiotics and the selective pressure resulting from the excessive, clinically unnecessary, use of antibiotics contributes to the emergence of antibiotic resistance (AMR) that is a growing global health concern. The spread of infections resistant to the currently available antibiotics could contribute to a death every three seconds globally by 2050 [1]. Currently patients with infections caused by antibiotic-resistant bacteria are at high risk of long hospital stays, treatment failure, high mortality, and high health care costs [2].

The prevalence of multi drug resistant (MDR) Enterobacteriaceae, especially those which are resistant to β-lactams, has been increasing worldwide because these antibiotics are important therapeutic choices for treating infections in humans [3].

*K. pneumoniae* is a notorious bacterium, and by acquiring additional antimicrobial resistance, it could be the origin of increasing nosocomial infections. This species is known to be prone to MDR and hypervirulent strain emergence [18].

*K. pneumoniae* cause severe infections including liver abscess, pneumonia, and sepsis by the production of enzymes such as extended-spectrum β-lactamases (ESBLs) or plasmid-mediated AmpC β-lactamases (pAmpC) that are enzymes commonly isolated from Enterobacteriaceae [4]. Nowadays, enterobacteria producing ESBLs and β-lactamase enzymes are frequently isolated from nosocomial and community-acquired infections [5]. Therefore, acquired carbapenemase-encoding genes in enterobacteria constitute a real clinical concern for antimicrobial management. ESBL, pAmpC and the carbapenemases are largely produced by MDR enterobacteria such as *Klebsiella* spp isolates. The corresponding genes are located on plasmids and other mobile genetic elements [6]. This is alarming because these plasmids frequently
carry genes encoding resistance to other classes of drugs such as aminoglycosides, trimethoprim-sulfamethoxazole, fluoroquinolones, and can move horizontally among bacteria [7]. This is especially the case with *Klebsiella* spp which is one of the bacteria in need of development of new therapeutic compounds [8]. The objective of this study was to evaluate the level of antibiotic resistance and to perform the molecular characterization of clinical *K. pneumoniae* strains isolated from out-patients and in-patients admitted to different wards of the hospital in Oum El Bouaghi, Algeria.

**Methodology**

**Samples collection**

30 Bacterial isolates were provided by the laboratory of Microbiology of the hospital at Oum El Bouaghi, Algeria. They were isolated between December 2021-February 2022 from various clinical samples (urinary tract infections, hemoculture and pus) collected from out-patients and in-patients admitted to different wards of the hospital.

The isolates were grown on MacConkey agar (BioMerieux, Marcy l’Etoile, France) for 24 hrs at 37 ± 1 °C. The isolates were identified using biochemical tests, and the identification was confirmed by mass spectrometry using VITEK® MS (BioMerieux, Marcy l’Etoile, France). VITEK® MS PRIME is a Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometer. The instrument analyzes material from microbial cultures to identify the microorganism. The samples were exposed to multiple laser shots inside VITEK® MS PRIME [1], and this was designed to incorporate additional benefits and enhance the use of MALDI-TOF technology [1].

**Antimicrobial susceptibility testing**

Antimicrobial drug susceptibility was determined using the disc diffusion method on Mueller-Hinton agar and interpreted according to the recommendations of Antibiogram Committee French Society for Microbiology (http://www.sfm-microbiologie.org). The antimicrobial agents tested were: β-lactamins (10µg of ampicillin, 30µg of amoxicillin-clavulanic acid, 10µg of imipenem and 30µg of cefotaxime), cyclines (30µg of tetracycline), sulfonamides (25µg of tri-methoprim-sulfamethoxazole), aminoglycosides (10µg of gentamicin), rifamycines (5µg of rifampicin), and quinolones (30µg of nalidixic acid and 5µg of ciprofloxacin).

The screening of ESBL production was performed by the double-disc synergy test (DDST) between clavulanic acid and third generation cephalosporins (cefotaxime, ceftazidime, aztreonam and cefepime) [9]. The test was considered positive when a “champagne cork” aspect was observed.

**DNA sequencing**

Genomic DNA was extracted using a QIAxtractor (Qiagen, Valencia, CA), and library preparation was performed by using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) according to the Illumina protocol. The libraries were sequenced with the Illumina MiSeq platform (2x 300-bp paired-end reads) with a minimum of mapped-reads depth of 40-fold.

Contaminant searches and molecular identification were performed for each sample using the centrifuge. This method performs abundance analyses at all taxonomic levels (e.g. strain, species, genus) [10]. Quality control metrics were examined across the whole collection as a batch report to ensure a mean read base-pair quality score of Q ≥ 20 and a read length of 70% of the original read length. Quality-filtered Illumina reads were assembled using Unicycler [11]. Contigs were annotated using Bakta.

**Gene annotation**

To define the presence of specific genes and their alleles, we used ARIBA [12], DIAMOND [13], and the following databases: Multilocus sequence typing (MLST) database, serotypeFinder O:H typing database, the fimH typing database, and curated databases of AMR genes/Single Nucleotide Polymorphism (SNPs) including Res-Finder, NDARO, and CARD.

**Comparative genomic analysis**

The filtered whole genome sequencing (WGS) reads were aligned against *K. pneumoniae* core genome (https://www.cgmlst.org) to call SNPs using BactSNP [14]. A maximum-likelihood phylogeny was then generated by RAxML-ng v 0.9 [15] based on the resulting core genome alignment filtered for recombination using Gubbins v 2.2 [16].

**Results**

**Multilocus sequence typing (MLST) and clonality analysis**

According to data of MLST, all isolates belonged to the sequence type ST307. Clonality analysis performed by cgMLST on mean 2145635 bases (https://microreact.org/project/gekjDYwFLTkanVvTG Bzwoi-confile) showed that the most similar isolates 03 and 13 (ST307) differ by 39 SNPs. The isolates did not
therefore emerge as a recent clonal spread but probably correspond to an endemic reservoir of ST307.

**Discussion**

The World Health Organization (WHO) has recommended that countries should develop AMR surveillance programs to collect and integrate antimicrobial use.

Our genomic data analysis, presented in Table 1, confirmed that all *K. pneumoniae* strains (n = 4) are ESBLs. A coexistence of three ESBL genes (*CTX-M, TEM, and SHV*) was found in two strains (S03 and S13), and this was already reported in Algeria [19], and in other countries [20]. Association of ESBL genes lead to selection of resistance genes in hospitals as well as in other sectors. In addition, the presence of multiple β-lactamase genes with several variants in *K. pneumoniae* isolates would also be the cause of MDR and other extremely drug resistant (XDR) strains emergence [21]. These may lead to high human mortality rates when it comes to clinical infections due to failures of antimicrobial therapies [22]. Both bla*SHV* and bla*CTX-M* genes are present in the two strains (S01 and S09) analyzed in *K. pneumoniae*. The coexistence of bla*SHV* and bla*CTX-M* genes was also observed in previous studies conducted in Algeria [23].

Through this investigation, the *CTX-M* genes have been identified in all *K. pneumoniae* (S01, S03, S09, and S13); bla*CTX-M-15* type variant (CTX-M-1 group) in particular is a well distributed strain throughout the world, including Algeria [24].

A bla*SHV-11* gene was found in S01 and S09 strains that procures a broad-spectrum resistance to antibiotics in the *K. pneumoniae* strain. Two strains (S03 and S13) harboring the bla*SHV28* and the bla*TEM-1* genes have been identified, and have already been a subject of study in Algeria [23]. No data was available on the functional aspect of variant gene bla*TEM-1*.

Hyperproduction of TEM-1 β-lactamase mediated by the promoter *Pa/Pb* which was detected in two *K. pneumoniae* strains (S03 and S13) has never been addressed in Algeria, and according to other studies bla*TEM-1(Pa/Pb)* is only responsible of high resistance to piperacillin-tazobactam (TZP) [25].

The OXA gene was found present in three strains of *K. pneumoniae*. The OXA-1 gene was also detected in Algeria [26]. OXA-1 variant gene was discovered in coexistence with ESBL genes. This result is consistent with previous studies that showed that these genes are commonly present in combination with *CTX-M-15, SHV-1, TEM-1* β-lactamases, PMQR (plasmid mediated quinolones resistance) determinants, and aac(6’)-Ib-cr in a community of *K. pneumoniae* strain [27].

Previous studies have demonstrated that NDM-5 offer greater resistance than NDM-1 genes [34]. From what we observed, the detection of New Delhi Metallo-β-lactamases genes of type NDM-5 in *K. pneumoniae* (S09) strains is considered as a first in Algeria, However the blaNDM-5 gene has been reported only in other clinical isolates such as *Acinetobacter baumannii* [28], *Escherichia coli* [29], and *Enterobacter cloacae* [30].

In contrast, in other countries the bla*NDM-5* gene has been identified mainly in clinical Enterobacteriaceae. The carbapenems represent the latest threat to public health especially in the case of nosocomial transmission of the blaNDM gene which has occurred in many countries [31]. However, in Algeria, NDM-5 gene was detected in *K. pneumoniae* from animal origins such as white storks [32]. This finding confirms that wildlife in Algeria could serve as reservoirs of MDR *K. pneumoniae*, in addition to its presence in fecal samples of companion animals [33].

**Table 1. Antibiotic resistance-encoding genes found in clinical K. pneumoniae strains.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>B-lactam resistance</th>
<th>B-lactam gene(s)</th>
<th>Aminoglycoside de gene(s)</th>
<th>Quinolone gene(s)</th>
<th>Sulfonamide gene(s)</th>
<th>Trimethoprim gene(s)</th>
<th>Cyclic gene(s)</th>
<th>Phenicol gene(s)</th>
<th>Fosfomycin gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em> (S1)</td>
<td>ESBL, IRP</td>
<td>bla<em>CTX-M-1</em></td>
<td>aac(3)-Ile aac(6’)-Ib-cr aadA11</td>
<td>qnrB1 gyrA(SK31) sul1 sul2</td>
<td>dfrA1 dfrB1 tetA (G)</td>
<td>floR catB3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (S3)</td>
<td>ESBL, IRP, NB-L</td>
<td>bla<em>CTX-M-1</em></td>
<td>aph(3”)-Ia aph(6”)-ld aph(3”)-Ib</td>
<td>qnrB1 gyrA(SK31) sul2</td>
<td>dfrA14 tetA TetR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (S9)</td>
<td>ESBL, NB-L, Cr</td>
<td>bla<em>CTX-M-1</em></td>
<td>aph(3”)-Ib aph(3”)-Ia aph(6”)-ld</td>
<td>qnrS1 sul2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (S13)</td>
<td>ESBL, IRP, NB-L, P</td>
<td>bla<em>CTX-M-1</em></td>
<td>aac(3)-Ile aac(6’)-Ib-cr aph(6”)-ld aph(3”)-Ib</td>
<td>qnrB1 gyrA(SK31) sul2</td>
<td>dfrA14 tet(A) TetR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

One finding of the present study is the existence of fluoroquinolone-resistant strain of *K. pneumoniae* carrying two plasmid mechanisms of resistance to quinolones: the *aac(6')-Ib-cr* variant gene harbored by two strains (S01 and S13) and the *qnrB1* gene present in three strains (S01, S03, and S13); these were also previously identified in Algeria [35]. One strain (S09) had acquired the *qnrS1* gene. This is the second report of *qnrS1* in ESBL-*K. pneumoniae* in Algeria [26]. It was previously described that *qnrB* was predominant among strains from Africa that *qnrS*. An association has been found between the production of blaCTX-M-15, *qnrB1* and/or *qnrS1* gene in all strains of *K. pneumoniae*, based on data from studies in Algeria [36] and in other countries [37] which report cases of *K. pneumoniae* harboring blaCTX-M-15 and *aac(6')-Ib-cr* [38].

Based on studies conducted in Algeria, we report for the first time the chromosomal mechanism of resistance to quinolones in three *K. pneumoniae* strains (S01, S03, and S13) mediated by the Ser83Leu (S83I) substitution in *gyrA* gene, which is consistent with previous studies from several countries [39]. The *gyrA* (S83I) gene has been identified in strains of *K. pneumoniae* carrying plasmid mechanisms especially the *qnrB1* gene. Thus, the appearance of chromosomal mutations in the genes encoding DNA gyrase can increase the level of resistance of the bacteria to quinolones [40].

Our *qnr* positive strains show resistance to aminoglycosides, cotrimoxazole, sulfonamide, trimethoprim, fosfomycin, and cycline. This could be explained by the fact that the plasmid support of quinolone resistance is present in a cassette as part of an integron carrying the other PMQR genes like blaCTXM-15, *qnrB* and the multiresistance genes to other antibiotics [41].

Few studies in Algeria have identified the detailed expression of resistance to aminoglycosides. Our data show that all the strains were resistant to aminoglycosides but with different gene expression levels, and this includes *aph(6)-Id*, *aac(3)-Ile*, *aac(6')-Ib-cr*, *aph(3'-)Ia*, and *aph(3')-Ib* genes. One strain (S01) of *K. pneumoniae* harbored genes of the type *aadA11*, and this was first detected in Algeria from clinical strains of *K. pneumoniae*. Among these identified genes, the most concerning is the *aac(6')-Ib-cr* variant gene [42]. All of aminoglycosides resistant genes discovered in this study have not been studied in Algeria, especially since most of them have been located in mobile elements (plasmids, integron, transposon and integrative conjugative element) [43].

This study is the first investigation in Algeria analyzing resistance to sulfonamides and tetracycline in clinical strains of *K. pneumoniae*. Resistance to sulfonamides in all *K. pneumoniae* strains (*n* = 4) is due to *sul1* and *sul2* genes. In addition, we identified resistance to trimethoprim by *dfrA1*, *a7*, *dfrA14*, and *dfrB1*. All the strains showed resistance to fosfomycin and carried a *fosA* gene. There were three strains that carried *tetA*, *tetG*, and *tetR* genes that lead to resistance to cyclines. The acquisition of resistance to fosfomycin and tetracycline by clinical isolates of *K. pneumoniae* sharing ESBL and/or carbapenemases represents a new threat that complicates the situation and shows that *K. pneumoniae* remains a very serious causative agent of therapy failure [44].

The most similar isolates S03 and S13 (ST307) differed only by 39 SNPs and both carried the ESBLs genes: *blaTEM-1*, *blaCTX-M-15*, *blaOXA-1*, *blaSHV-28*, and *blaTEM* (PaPb). They belong to the same strain ST-307 as indicated in Figure 1 that demonstrates the phylogenetic tree of multilocus sequence typing (MLST) of *K pneumoniae*. The ST307 clone has been previously detected in Algeria by [45]. Our results show that this clone was associated with three ESBLs (*blaCTX-M-15), *qnrB* and other genes. Several reports have showed that the origin of MDR ST307 *K. pneumoniae* was from clinical samples, as well as from other sources [57]. The WHO recently declared that the ST307 strain poses critical threat to public health It has also been concluded that ST307 *K. pneumoniae* can often carry transferable resistance-conferring genes.

**Figure 1.** Phylogenetic tree of multilocus sequence typing (MLST) clinical MDR *K. pneumoniae* isolates.

Numbers (01, 03, 09 and 13) correspond to the number of each strain of *Klebsiella pneumoniae*. 

**Table 1.** Antimicrobial resistance profile of *K. pneumoniae* clinical strains.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistance Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>100% R</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>50% R</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>100% R</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>100% R</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>100% R</td>
</tr>
<tr>
<td>Cycline</td>
<td>100% R</td>
</tr>
</tbody>
</table>

The resistance pattern of the strains is highly consistent with previous reports in Algeria [26].
against carbapenems and a variety of additional resistance and virulence determinants, in addition to integrative and conjugative elements, and phages. As a result, ST307 *K. pneumoniae* is emerging globally as an important vehicle for the dissemination of AMR determinants [58], and has been responsible for several global nosocomial and long-term care center outbreaks [59]. The MLST analysis indicated that ST307 consisted of one deep-branching lineage which contained the *gyrA* S83I mutation in the quinolone resistance determinant region (QRDR) that had global distribution [60].

**Conclusions**

The present study reports the first analysis and identification of the bacterial resistome of MDR clinical strains belonging to *Klebsiella pneumoniae* in Oum Bouaghi, Algeria. Our data demonstrate multiple clinical *K. pneumoniae* strains resistant to most common antibiotic families. This is also the first reported detection of *K. pneumoniae* producing blaN DM-5 gene in Algeria. Control of antimicrobial resistance requires monitoring and surveillance of the level of emergence of resistant bacteria, as well as the resistance genes and their location. These measures can limit the presence of new diverse AMR encoding genes that could be a source of emergence of new human pathogens in the future.

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**References**


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