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# Resistance to aminoglycoside and quinolone drugs among *Klebsiella pneumoniae* clinical isolates from northern Jordan

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ABSTRACT

This study aimed to identify phenotypic and genotypic aminoglycoside and quinolone non-susceptibility and the prevalence of aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance genes among *K. pneumoniae* clinical isolates from northern Jordan. *K. pneumoniae* isolates (n = 183) were tested for antimicrobial susceptibility using the Kirby-Bauer disk diffusion method. The double-disk synergy test was used for the detection of the extended-spectrum beta-lactamase phenotype. Polymerase chain reaction was used to detect genes encoding aminoglycoside-modifying enzyme (*aac (3′)-II*, *aac (6′)-II*, *aac (6′)-Ib*, *ant (3″)-I*, *aph (3′)-VI*, *armA*, and *rmtB*), and plasmid-mediated quinolone resistance (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *acc(6′)-Ib–cr*, *qepA*, and *oqxAB)* genes. Multi-locus sequence typing was used to elucidate the genetic diversity of selected isolates. The non-susceptibility percentages to aminoglycosides and quinolones were 65.0 % and 61.7 %, respectively. The most frequent aminoglycoside-modifying enzyme gene was *ant (3″)-I* at 73.8 %, followed by *aac (6′)-Ib* at 25.1 %, *aac (3′)-II* at 17.5 %, *aph (3′)-VI* at 12.0 %, *armA* at 9.8 %, and *rmtB* at 0.5 %. *Aac (6′)-II* was not detected among the isolates. The most frequent plasmid-mediated quinolone resistance gene was *oqxAB* at 31.7 %, followed by *qnrS* at 26.2 %, *qnrB* at 25.7 %, and *aac(6′)-Ib–cr* at 25.7 %. *QnrA*, *qnrD*, *qebA*, and *qnrC* were not detected among the isolates. *Aac (3′)-II*, *aac (6′)-Ib*, *aph (3′)-VI*, *armA*, *qnrB*, *qnrS*, and *acc(6′)-Ib–cr* were significantly associated with non-susceptibility to aminoglycosides, quinolones, and beta-lactams. Among 27 randomly selected *K. pneumoniae* isolates, the most common sequence type was ST2096, followed by ST348 and ST1207. Overall, 19 sequence types were observed, confirming a high level of genetic diversity among the isolates. High percentages of non-susceptibility to the studied antimicrobials were found and were associated with the presence of several resistance genes. Similar studies should be periodically carried out to monitor changes in the prevalence of resistance phenotypes and genotypes of isolates.

## 1. Introduction

*Klebsiella pneumoniae* has developed resistance to many classes of antimicrobials and is one of the most common causes of hospital-acquired infections [1,2]. The overuse of antimicrobials, such as aminoglycosides and quinolones, led to the emergence of resistance among clinical *K. pneumoniae* isolates, which increases the rates of morbidity and mortality among patients. Aminoglycosides are broad-spectrum antimicrobials that include agents such as gentamicin, tobramycin, amikacin, plazomicin, streptomycin, and

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neomycin. The agents within this class act by suppressing bacterial polypeptide synthesis, which leads to bacterial cell death [3]. Development of resistance to aminoglycosides has been observed among *Pseudomonas aeruginosa* and other Gram-negative bacilli [4]. Several mechanisms may be responsible for mediating resistance, such as decreased intracellular drug concentration, target modification, and enzymatic drug modification [5]. Enzymatic drug modification includes aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs) [5]. APH, AAC, and ANT enzymes are collectively referred to as aminoglycoside-modifying enzymes (AMEs). Genes encoding the common AMEs in *K. pneumoniae* include *aac (3′)-II, aac (6′)-II, aac (6′)-Ib, ant (3″)-I and aph (3′)-VI* [6]. Additionally, *armA*, and *rmtB* play a role in mediating resistance against aminoglycosides [6].

On the other hand, quinolones are synthetic antimicrobials that inhibit DNA synthesis by converting their targets (DNA gyrase and topoisomerase IV) into enzymes that fragment the bacterial chromosome. Resistance to quinolones among bacteria can be mediated by chromosomal- and plasmid-mediated mechanisms [7]. The mechanisms of plasmid-mediated quinolone resistance include target alteration by Qnr proteins, quinolone modifying enzymes, and quinolone efflux pumps [7]. Qnr proteins protect the DNA gyrase and topoisomerase IV from quinolones. These proteins are encoded by plasmid genes *qnrA, qnrB, qnrC, qnrD, qnrS,* and *qnrVC* [8]. These genes in combination with mutations in chromosomal genes, allow bacteria to develop strong resistance to quinolones. The quinolone modifying enzymes such as the aminoglycoside acetyltransferase (encoded by *acc(6′)-Ib -cr*) acetylate the quinolone drug making it less effective. The quinolone efflux pumps prevent the accumulation of quinolones in the bacterial cell. These pumps are encoded by several plasmid genes, including *qepA* and *oqxAB* [8]. The plasmid-mediated quinolone resistance (PMQR) genes are reported among many members of the *Enterobacteriaceae*, such as *K. pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Escherichia coli*, *P. aeruginosa*, *Acinetobacter baumannii*, among others [9].

The prevalence of genes encoding AMEs and PMQR among aminoglycoside and quinolone-resistant *K. pneumoniae* clinical isolates has been reported for many countries [10–19]. To our knowledge, this study is the first to report the prevalence of AMEs and PMQR genes among *K. pneumoniae* from northern Jordan. The specific aims of this study include the determination of the antibiogram of clinical *K. pneumoniae* isolates from northern Jordan, the prevalence of AMEs and PMQR genes among the isolates, the associations between genotypes and antimicrobial phenotypes, and the identification of the sequence types of a subset of isolates. Misuse of antimicrobials is common in Jordan [20]. Hence, we expect that a significant percentage of clinical *K. pneumoniae* isolates will exhibit resistance to quinolones and aminoglycosides, as was previously reported [21–23]. We hypothesize that several genes encoding AMEs and PMQR could be prevalent among aminoglycoside and quinolone-resistant clinical *K. pneumoniae* isolates obtained from Jordan.

## 2. Materials and methods

### 2.1. Bacterial isolates

Approval for the study was granted by the IRB committee of Jordan University of Science and Technology (Approval ID 26/144/2021). One hundred and eighty-three non-replicate *K. pneumoniae* isolates were collected form the microbiology laboratories of several hospitals from Northern Jordan over a seven-months period (July–October 2021 and January–April 2022). All clinical isolates were identified using the VITEK 2 System (bioMérieux Inc., USA) at the respective hospitals. Judgmental sampling was utilized during isolates' collection. Any isolate identified as *Klebsiella pneumoniae* at the respective hospitals were included in the study regardless of clinical sample type, or patient gender or age. Isolates were obtained from primary cultures following identification. Information regarding the isolates was obtained from medical records. The isolates were cultured directly on Mueller-Hinton agar (MHA) and immediately transferred to the research laboratory for overnight incubation at 37 °C. Next, isolates were grown in LB broth overnight at 37 °C and stored at −80 °C with 17 % glycerol for later use. Confirmation of species identity was performed using PCR via detection of the *K. pneumoniae* specific *phoE* as described below.

### 2.2. Antimicrobial susceptibility testing

Isolates from frozen cultures were used to inoculate LB broth tubes and the tubes were cultured overnight at 37 °C. The broth cultures were adjusted to a turbidity equivalent to 0.5 McFarland standard and were used to inoculate Mueller Hinton Agar plates (using cotton swabs) for antimicrobial susceptibility testing against several classes, including quinolones and aminoglycosides using the Kirby-Bauer disk diffusion method based on CLSI (M100, 2022) performance standards for antimicrobial susceptibility testing. The following antimicrobial disks were used: cefepime (30 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), kanamycin (30 μg), netilmicin (30 μg), streptomycin (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), lomefloxacin (10 μg), norfloxacin (10 μg), and ofloxacin (5 μg). The zone interpretation criteria were per the CLSI antimicrobial breakpoints (2022).

The double disk synergy test was used to detect ESBL production according to CLSI guidelines (2022). A disk of amoxicillin-clavulanate (20/10 μg) along with aztreonam (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefpodoxime (10 μg), and ceftriaxone (30 μg) disks, were used. An MHA plate was inoculated with each isolate as described above. Next, an amoxicillin-clavulanate disk was placed in the center of the plate, and aztreonam, cefotaxime, ceftazidime, cefpodoxime, ceftriaxone disks were placed 25 mm (center to center) from the amoxicillin-clavulanate disk. After overnight incubation at 37 °C, any distortion or increase in the zone of inhibition (i.e., augmentation of inhibition) toward the amoxicillin-clavulanate acid disk was considered a positive result for the ESBL production [24]. Isolates were determined to have the multidrug-resistant (MDR) phenotype upon demonstration of nonsusceptibility toward at least one agent among three or more antimicrobial classes. Isolates were determined to have the extensively drug-resistant (XDR) phenotype upon demonstration of nonsusceptibility toward at least one agent among all antimicrobial classes tested except two.

*E. coli* ATCC 25922, *Klebsiella pneumoniae* BAA-2146, *Klebsiella pneumoniae* BAA-1705, *Klebsiella pneumoniae* BAA-1706, were used as controls during antimicrobial susceptibility and ESBL testing.

### 2.3. Molecular studies

DNA was extracted using a commercial kit (Patho Gene-spin DNA/RNA Extraction Kit, iNtRON Biotechnology, South Korea) following the manufacturer's recommendations. The extracted DNA samples were analyzed using a Nanodrop spectrophotometer to determine DNA purity and concentration. All DNA samples were stored at −20 °C until used for PCR.

For verification of *K. pneumoniae* isolates' identities, the *phoE* gene specific to *K. pneumoniae* was amplified using PCR as previously described [25]. The components of each partial amplification reaction were: 1 μL template (10 ng/ml), 2 μL primers (10 μmol/L), 5 μL 5x master mix (Ready-to load 5X Master Mix, myPOLS Biotech, LOT 061021KMC, Germany), and 17 μL nuclease-free water. The total PCR volume was 25 μL. Confirmation of amplified genes was done via sequencing. Primers and PCR conditions are indicated in Supplementary Table S1.

All *K. pneumoniae* isolates were screened for 7 genes encoding AMEs, namely *aac (3′)-II, aac (6′)-II, aac (6′)-Ib, ant (3″)-I and aph (3′)-VI, armA,* and *rmtB* [6]. PCR conditions were as previously described [11,26]. Each PCR was performed at a 25 μL final reaction volume, including: 1 μL of extracted DNA, 5 μL of 5x master (Ready-to load 5X Master Mix, myPOLS Biotech, LOT 061021KMC, Germany), 1 μL of forward primer (10 μmol/L), 1 μL of reverse primer (10 μmol/L), and 17 μL of nuclease free water. Confirmation of amplified genes was done via sequencing. The primers, expected amplicon sizes, and PCR conditions are listed in Supplementary Table S2.

All *K. pneumoniae* isolates were screened for 5 quinolone resistance genes (*qnrA*, *qnrB*, *qnrC*, *qnrD,* and *qnrS*), one quinolone-modifying enzyme gene (*aac(6′)-Ib–cr),* and for *oqxAB*, and *qebA*, by two multiplex PCRs. The PCR conditions were as follow: initial denaturation at 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 63 °C for 90 s and 72 °C for 90 s; followed by a final extension at 72 °C for 10 min as previously described [27]. Each PCR was performed at a final reaction volume of 25 μL, including: 1 μL of extracted DNA, 5 μL 5x master mix (Ready-to load 5X Master Mix, myPOLS Biotech, LOT 061021KMC, Germany), 1 μL of each forward primer (10 μmol/L), 1 μL of each reverse primer (10 μmol/L), and nuclease-free water (7 μL for reaction 1 and 15 μL for reaction 2). Confirmation of amplified genes was done via sequencing. The primers used and the expected amplicon sizes are listed in Supplementary Table S3.

Multi-locus sequence typing (MLST) analysis of 27 of the 183 isolates was performed using Pasteur's MLST scheme. These isolates were chosen to represent samples with a low number of resistance genes, a moderate number of resistance genes, and a high number of resistance genes. Seven housekeeping genes, i.e., *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*, were used to characterize *K. pneumoniae*. Amplification conditions and primers were from https://bigsdb.pasteur.fr/klebsiella/primers_used.html, as indicated in Supplementary Table S4. For each isolate, the seven amplified gene fragments were sent to Macrogen Biotechnology Co. (South Korea) for sequencing. The primers used for sequencing the amplified *phoE*, *rpoB*, and *gapA* fragments were the same primers used for PCR. While universal sequencing primers were used for sequencing the amplified *infB, tonB, mdh,* and *pgi* fragments (F**:** GTTTTCCCAGTCACGACGTTGTA, R**:** TTGTGAGCGGATAACAATTTC). The sequencing results were compared with known alleles to identify the isolate's specific allelic profile. Lastly, the sequence type was obtained by combining the allele numbers for the seven genes. The Institute Pasteur website, Klebsiella locus/sequence definitions (pasteur.fr), was used for querying the sequence and finding the ST by locus combination.

### 2.4. Statistical analyses

The statistical package for social sciences (SPSS ver. 26, IBM, Armonk, NY, USA) software was used to generate tables and cross-tabulations. Pearson's chi-squared test was used to identify associations between variables. A $p$ value $< 0.05$ was considered significant.

**Table 1**
Isolates' information.

| Criteria | | n (%) |
|---|---|---|
| Source | Blood | 19 (10.4) |
| | Cerebrospinal fluid | 3 (1.6) |
| | High vaginal swab | 3 (1.6) |
| | Pus | 5 (2.7) |
| | Sputum | 3 (1.6) |
| | Tip of central line | 1 (0.5) |
| | Urine | 145 (79.2) |
| | Wound | 4 (2.2) |
| Gender | Female | 120 (65.6) |
| | Male | 63 (34.4) |

## 3. Results

### 3.1. Antimicrobial susceptibility

The 183 *K. pneumoniae* isolates were from different hospitals (data not shown) and different clinical specimens from northern Jordan. Most isolates were from urine, followed by blood, and most were from females (Table 1). The percentages of susceptible isolates and non-susceptible isolates (intermediately susceptible + resistant) against each antibiotic are shown in Table 2. The highest non-susceptibility was against amoxicillin-clavulanate, followed by cefpodoxime. The highest susceptibility was against netilmicin, followed by amikacin. More than half of the isolates were susceptible to aminoglycoside antibiotics, except for kanamycin. More than half of the isolates were susceptible to quinolone antibiotics, except for ciprofloxacin. Among the isolates, 31.7 % (58/183) were ESBL producers. Upon considering nonsusceptibility to any of the aminoglycoside or quinolone drugs as nonsusceptibility to either class, the non-susceptibility percentages to each class were 65.0 % (119/183) and 61.7 % (113/183), respectively. Supplementary Table S5 lists the full antimicrobial susceptibility results for the isolates. Interestingly, isolates from males demonstrated higher non-susceptibility phenotypes, than those from females, for 11 out of the 18 tested antibiotics (Table S6). The MDR and XDR phenotypes were observed among 62.3 % (113/183) and 7.7 % (14/183) of the isolates, respectively.

### 3.2. Prevalence of genes encoding AMEs and PMQR

For each isolate, 10 PCR amplifications were done for the detection of *phoE* and genes encoding AMEs and PMQR. Table 3 lists the frequency of detected genes. Among genes encoding AMEs, the most frequent was *ant (3″)-I* at 73.8 %, followed by *aac (6′)-Ib* at 25.1 %, while *aac (6′)-II* was not detected among the isolates. Among the genes encoding PMQRs, the most frequent was *oqxAB* at 31.7 %, followed by *qnrS* at 26.2 %. *QnrA, qnrD, qebA,* and *qnrC* genes were not detected among the isolates. Supplementary Table S7 lists the full PCR results for all isolates. The isolates with five resistance genes or more represented 15.3 % of the isolates, while only one isolate had seven resistance genes, as indicated in Table 4.

### 3.3. Association of genes encoding AMEs and PMQR with antimicrobials' non-susceptibility phenotypes

*Aac (3′)-II, aac (6′)-Ib, aph (3′)-VI,* and *armA* were significantly associated with non-susceptibility to aminoglycosides (kanamycin, gentamicin, tobramycin, and netilmicin) (Table 5, and Supplementary Tables S8 and S9). *QnrB, qnrS,* and *acc(6′)-Ib–cr* were significantly associated with non-susceptibility to quinolones (ciprofloxacin, levofloxacin, and lomefloxacin) (Table 6 and Supplementary Table S10). *Aac (3′)-II, aac (6′)-Ib, aph (3′)-VI,* and *armA* were also significantly associated with non-susceptibility to quinolones and beta-lactams, while *qnrB, qnrS,* and *acc(6′)-Ib–cr* were significantly associated with non-susceptibility to aminoglycosides and beta-lactams. *Aac (3′)-II, aph (3′)-VI, armA, qnrB, qnrS,* and *acc(6′)-Ib–cr* were significantly associated with the ESBL phenotype (Tables 5 and 6, and Supplementary Tables S8–S10).

### 3.4. Co-association of genes encoding AMEs and PMQR

The co-association of AME genes and PMQR genes among the isolates is demonstrated in Table 7. *QnrB* was significantly co-

**Table 2**
Antimicrobial susceptibility of the isolates.

| Antimicrobial Class | Antimicrobial Agent | I | R | NS (I + R) | S |
|---|---|---|---|---|---|
| | | n (%) | n (%) | n (%) | n (%) |
| Aminoglycosides | Kanamycin | 21 (11.5) | 80 (43.7) | 101 (55.2) | 82 (44.8) |
| | Gentamicin | 0 (0) | 45 (24.6) | 45 (24.6) | 138 (75.4) |
| | Amikacin | 11 (6.0) | 21 (11.5) | 32 (17.5) | 151 (82.5) |
| | Tobramycin | 1 (0.5) | 50 (27.3) | 51 (27.9) | 132 (72.1) |
| | Netilmicin | 6 (3.3) | 24 (13.1) | 30 (16.4) | 153 (83.6) |
| | Streptomycin | 11 (6.0) | 61 (33.3) | 72 (39.3) | 111 (60.7) |
| Quinolones | Ciprofloxacin | 42 (23.0) | 66 (36.1) | 108 (59.0) | 75 (41.0) |
| | Levofloxacin | 35 (19.1) | 42 (23.0) | 77 (42.1) | 106 (57.9) |
| | Lomefloxacin | 24 (13.1) | 53 (29.0) | 77 (42.1) | 106 (57.9) |
| | Norfloxacin | 9 (4.9) | 36 (19.7) | 45 (24.6) | 138 (75.4) |
| | Ofloxacin | 2 (1.1) | 41 (22.4) | 43 (23.5) | 140 (76.5) |
| 4th generation cephalosporin | Cefepime | 12 (6.6) | 77 (42.1) | 89 (48.6) | 94 (51.4) |
| Beta-Lactam and beta-lactamase inhibitor | Amoxicillin-clavulanate | 59 (32.2) | 73 (39.9) | 132 (72.1) | 51 (27.9) |
| Monobactam | Aztreonam | 20 (10.9) | 57 (31.1) | 77 (42.1) | 106 (57.9) |
| 3rd generations cephalosporin | Cefpodoxime | 5 (2.7) | 98 (53.6) | 103 (56.3) | 80 (43.7) |
| | Ceftazidime | 32 (17.5) | 51 (27.9) | 83 (45.4) | 100 (54.6) |
| | Ceftriaxone | 3 (1.6) | 87 (47.5) | 90 (49.2) | 93 (50.8) |
| | Cefotaxime | 7 (3.8) | 90 (49.2) | 97 (53.0) | 86 (47.0) |

I: intermediate susceptibility. N: count. NS: non-susceptible. R: resistant. S: susceptible.

**Table 3**
The frequency of detected aminoglycoside modifying enzymes and **plasmid-mediated quinolone resistance** genes.

| Gene | | n (%) |
|---|---|---|
| *K. pneumoniae* specific gene | *phoE* | 183 (100.0) |
| aminoglycoside modifying enzyme genes | *aac (3′)-II* | 32 (17.5) |
| | *aac (6′)-Ib* | 46 (25.1) |
| | *aac (6′)-II* | 0 (0) |
| | *ant (3″)-I* | 135 (73.8) |
| | *aph (3′)-VI* | 22 (12.0) |
| | *armA* | 18 (9.8) |
| | *rmtB* | 1 (0.5) |
| **plasmid-mediated quinolone resistance** genes | *qnrA* | 0 (0) |
| | *qnrD* | 0 (0) |
| | *qnrB* | 47 (25.7) |
| | *qnrS* | 48 (26.2) |
| | *oqxAB* | 58 (31.7) |
| | *aac(6′)-Ib–cr* | 47 (25.7) |
| | *qebA* | 0 (0) |
| | *qnrC* | 0 (0) |

**Table 4**
Isolates with the highest number of resistance genes.

| Isolate ID | Source | Gender | Date | Resistance genes count | Resistance genes |
|---|---|---|---|---|---|
| 22 | Cerebrospinal fluid | Male | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, qnrS* |
| 23 | Urine | Female | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 26 | Cerebrospinal fluid | Male | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 36 | Wound swab | Male | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, aac(6′)-Ib–cr* |
| 41 | Blood | Male | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, aac(6′)-Ib–cr* |
| 43 | Urine | Female | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, aac(6′)-Ib–cr* |
| 45 | Urine | Male | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 48 | Wound swab | Male | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 49 | Urine | Female | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, aac(6′)-Ib–cr* |
| 50 | Pus | Male | March 2022 | 6 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, qnrS, aac(6′)-Ib–cr* |
| 51 | Blood | Female | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 55 | Blood | Male | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, aac(6′)-Ib–cr* |
| 70 | Tip of central line | Male | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, aac(6′)-Ib–cr* |
| 75 | Blood | Female | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, oqxAB, aac(6′)-Ib–cr* |
| 78 | Blood | Female | March 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, oqxAB, aac(6′)-Ib–cr* |
| 83 | Urine | Male | March 2022 | 5 | *ant (3″)-I, aph (3′)-VI, armA, oqxAB, aac(6′)-Ib–cr* |
| 92 | Urine | Female | March 2022 | 6 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, oqxAB, aac(6′)-Ib–cr* |
| 102 | Blood | Male | March 2022 | 6 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, armA, qnrS, oqxAB* |
| 107 | Pus | Male | March 2022 | 5 | *aac (3′)-II, aac (6′)-Ib, aph (3′)-VI, qnrS, aac(6′)-Ib–cr* |
| 108 | Urine | Male | April 2022 | 5 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, qnrB, qnrS* |
| 112 | Blood | Male | April 2022 | 6 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, qnrB, qnrS, oqxAB* |
| 118 | Urine | Male | July 2021 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrS, aac(6′)-Ib–cr* |
| 160 | Urine | Male | September 2021 | 6 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, oqxAB, aac(6′)-Ib–cr* |
| 167 | Urine | Male | September 2021 | 5 | *aac (6′)-Ib, ant (3″)-I, qnrB, oqxAB, aac(6′)-Ib–cr* |
| 171 | Urine | Male | September 2021 | 7 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrB, qnrS, oqxAB, aac(6′)-Ib–cr* |
| 174 | Urine | Male | September 2021 | 5 | *aac (3′)-II, aac (6′)-Ib, qnrB, oqxAB, aac(6′)-Ib–cr* |
| 180 | Urine | Male | September 2021 | 5 | *aac (3′)-II, aac (6′)-Ib, ant (3″)-I, qnrS, aac(6′)-Ib–cr* |
| 181 | Urine | Female | September 2021 | 6 | *aac (6′)-Ib, ant (3″)-I, aph (3′)-VI, qnrS, oqxAB, aac(6′)-Ib–cr* |

associated with *aac (3′)-II*. Significant co-associations were also seen between each of the following pairs (*qnrB* and *aac (6′)-Ib*), (*acc (6′)-Ib–cr* and *aac (3′)-II*), (*acc(6′)-Ib–cr* and *aac (6′)-Ib*), (*acc(6′)-Ib–cr* and *aph (3′)-VI*), and (*acc(6′)-Ib–cr* and *armA*). On the other hand, significant gene independence was seen between *qnrB* and *armA*.

### 3.5. Molecular typing of *K. pneumoniae* isolates

Multi-locus sequence typing was performed to show the differences between 27 isolates. The isolates belonged to 19 sequence types. Seven isolates belonged to the same sequence type (ST2096). ST348 and ST1207 were detected twice. While ST4, ST15, ST39, ST111, ST193, ST383, ST432, ST556, ST661, ST1198, ST1801, ST1999, ST2299, ST2343, ST2648, and ST3769, were detected once. Table 8 and Supplementary Table S11 show the ST numbers and the allelic profiles for the studied isolates.

**Table 5**
Association *p* values of genes encoding aminoglycoside modifying enzymes with non-susceptibility phenotypes.

| Non-susceptible Phenotype/Gene | *aac (3′)-II* | *aac (6′)-Ib* | *ant (3″)-I* | *aph (3′)-VI* | *armA* | *rmtB* |
|---|---|---|---|---|---|---|
| ESBL | < **0.001** | 0.210 | 0.939 | **0.004** | **0.012** | 0.141 |
| Kanamycin | < **0.001** | < **0.001** | 0.868 | **0.007** | **0.042** | 0.266 |
| Gentamicin | < **0.001** | < **0.001** | 0.138 | < **0.001** | < **0.001** | 0.567 |
| Amikacin | 0.414 | < **0.001** | 0.862 | < **0.001** | < **0.001** | 0.644 |
| Tobramycin | < **0.001** | < **0.001** | **0.044** | < **0.001** | **0.001** | 0.533 |
| Netilmicin | < **0.001** | < **0.001** | 0.396 | < **0.001** | < **0.001** | 0.657 |
| Streptomycin | < **0.001** | 0.507 | 0.321 | **0.008 * Inverse** | **0.010 * Inverse** | 0.213 |
| Ciprofloxacin | **0.001** | < **0.001** | **0.004** | 0.163 | 0.487 | 0.403 |
| Levofloxacin | **0.003** | < **0.001** | **0.002** | **0.002** | **0.026** | 0.239 |
| Lomefloxacin | **0.029** | < **0.001** | 0.077 | **0.002** | **0.026** | 0.393 |
| Norfloxacin | **0.001** | < **0.001** | **0.024** | < **0.001** | < **0.001** | 0.567 |
| Ofloxacin | **0.040** | < **0.001** | **0.004** | < **0.001** | < **0.001** | 0.578 |
| Cefepime | < **0.001** | < **0.001** | 0.431 | **0.016** | **0.035** | 0.303 |
| Amoxicillin-clavulanate | **0.003** | < **0.001** | 0.373 | **0.036** | 0.095 | 0.533 |
| Aztreonam | **0.001** | < **0.001** | 0.455 | **0.008** | **0.026** | 0.239 |
| Cefpodoxime | **0.002** | < **0.001** | 0.996 | **0.002** | 0.053 | 0.377 |
| Ceftazidime | **0.001** | < **0.001** | 0.35 | **0.022** | 0.056 | 0.271 |
| Ceftriaxone | < **0.001** | < **0.001** | 0.838 | **0.005** | **0.039** | 0.308 |
| Cefotaxime | < **0.001** | < **0.001** | 0.851 | **0.001** | **0.027** | 0.345 |

*P* values were obtained via the Pearson's chi-squared test. Values in bold are statistically significant. ESBL: extended-spectrum beta-lactamase. "Inverse" indicates significant independence.

**Table 6**
Association *p* values of genes encoding plasmid-mediated quinolone resistance with non-susceptibility phenotypes.

| Non-susceptible Phenotype/Gene | *qnrB* | *qnrS* | *oqxAB* | *aac(6′)-Ib–cr* |
|---|---|---|---|---|
| ESBL | **0.010** | < **0.001** | 0.637 | **0.003** |
| Kanamycin | **0.006** | **0.001** | 0.752 | < **0.001** |
| Gentamicin | < **0.001** | 0.754 | 0.641 | < **0.001** |
| Amikacin | 0.922 | 0.788 | 0.953 | < **0.001** |
| Tobramycin | < **0.001** | 0.606 | 0.68 | < **0.001** |
| Netilmicin | 0.132 | 0.693 | 0.827 | < **0.001** |
| Streptomycin | **0.003** | < **0.001** | 0.953 | 0.118 |
| Ciprofloxacin | < **0.001** | < **0.001** | 0.803 | < **0.001** |
| Levofloxacin | < **0.001** | **0.008** | 0.247 | < **0.001** |
| Lomefloxacin | **0.005** | **0.003** | 0.247 | < **0.001** |
| Norfloxacin | **0.003** | 0.482 | 0.080 | < **0.001** |
| Ofloxacin | **0.017** | 0.612 | **0.044** | < **0.001** |
| Cefepime | 0.161 | < **0.001** | 0.308 | < **0.001** |
| Amoxicillin-clavulanate | **0.002** | 0.373 | 0.443 | < **0.001** |
| Aztreonam | **0.033** | **0.001** | 0.608 | < **0.001** |
| Cefpodoxime | < **0.001** | **0.018** | 0.909 | < **0.001** |
| Ceftazidime | < **0.001** | **0.015** | 0.677 | < **0.001** |
| Ceftriaxone | **0.046** | **0.002** | 0.880 | < **0.001** |
| Cefotaxime | **0.001** | **0.004** | 0.813 | < **0.001** |

*P* values were obtained via the Pearson's chi-squared test. Values in bold are statistically significant. ESBL: extended-spectrum beta-lactamase.

## 4. Discussion

Strains with multiple resistance genes are a serious problem as they can easily spread this resistance. Due to the widespread use of quinolones and aminoglycosides, the resistance of *K. pneumoniae* and other bacteria has been rapidly increasing worldwide in the clinical setting. Therefore, it is crucial to continuously monitor the spread of *K. pneumoniae* and its resistance genes. This would enable setting of new policies to control its spread and lessen its clinical impact.

In this study we collected clinical *K. pneumoniae* isolates. The majority were from urinary tract infections likely due to the proximity of the anal opening to the urinary tract. Almost two-thirds of the isolates were from females, likely due to the higher frequency of urinary tract infections among females than males. The drug with the highest non-susceptibility was amoxicillin-clavulanate (72.1 %), followed by aminoglycosides (65.0 %), and quinolones (61.7 %). Among the aminoglycosides, the highest non-susceptibility was against kanamycin (55.2 %). Among the quinolones, the highest non-susceptibility was against ciprofloxacin (59.0 %). Non-susceptibility against cefpodoxime was highest among the beta-lactam antimicrobials (56.3 %).

As indicated in Supplementary Table S6, *K. pneumoniae* isolated from males showed higher non-susceptibility percentages to several aminoglycosides, quinolones, and beta-lactams, than those from females. Hence, gender could be a factor in determining appropriate prophylactic therapy of *K. pneumoniae* in Jordan. Previous studies showed conflicting results regarding gender. Some studies found that isolates from males had higher nonsusceptibility to antimicrobials [28,29]. While other studies reported the

**Table 7**
Co-association of genes encoding aminoglycoside modifying enzymes and plasmid-mediated quinolone resistance.

| Gene | | *qnrB* | | *qnrS* | | *oqxAB* | | *aac(6′)-Ib–cr* | |
|---|---|---|---|---|---|---|---|---|---|
| | | – | + | – | + | – | + | – | + |
| *aac (3′)-II* | – | 125 | 26 | 112 | 39 | 100 | 51 | 122 | 29 |
| | + | 11 | 21 | 23 | 9 | 25 | 7 | 14 | 18 |
| | *P* value | < **0.001** | | 0.788 | | 0.189 | | < **0.001** | |
| *aac (6′)-Ib* | – | 111 | 26 | 99 | 38 | 92 | 45 | 127 | 10 |
| | + | 25 | 21 | 36 | 10 | 33 | 13 | 9 | 37 |
| | *P* value | < **0.001** | | 0.424 | | 0.563 | | < **0.001** | |
| *ant (3″)-I* | – | 37 | 11 | 41 | 7 | 31 | 17 | 36 | 12 |
| | + | 99 | 36 | 94 | 41 | 94 | 41 | 100 | 35 |
| | *P* value | 0.610 | | 0.033 | | 0.519 | | 0.900 | |
| *aph (3′)-VI* | – | 116 | 45 | 119 | 42 | 111 | 50 | 126 | 35 |
| | + | 20 | 2 | 16 | 6 | 14 | 8 | 10 | 12 |
| | *P* value | 0.058 | | 0.906 | | 0.616 | | **0.001** | |
| *armA* | – | 118 | 47 | 119 | 46 | 111 | 54 | 127 | 38 |
| | + | 18 | 0 | 16 | 2 | 14 | 4 | 9 | 9 |
| | *P* value | **0.009* Inverse** | | 0.125 | | 0.363 | | **0.013** | |
| *rmtB* | – | 136 | 46 | 134 | 48 | 124 | 58 | 135 | 47 |
| | + | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |
| | *P* value | 0.088 | | 0.550 | | 0.495 | | 0.556 | |

"-": absent. "+": present. Values in bold are statistically significant. "Inverse" indicates significant independence.

**Table 8**
Isolates subjected to MLST analysis.

| Isolate ID | Source | Gender | Collection date | Sequence type (ST) |
|---|---|---|---|---|
| 1 | Urine | Male | January 2022 | 2648 |
| 15 | Urine | Female | February 2022 | 432 |
| 30 | Wound swab | Female | March 2022 | 348 |
| 34 | Blood | Male | March 2022 | 193 |
| 35 | Pus | Male | March 2022 | 2096 |
| 43 | Urine | Female | March 2022 | 2096 |
| 50 | Pus | Male | March 2022 | 39 |
| 67 | Urine | Male | March 2022 | 2343 |
| 69 | Urine | Male | March 2022 | 1198 |
| 77 | Urine | Female | March 2022 | 2299 |
| 78 | Blood | Female | March 2022 | 2096 |
| 79 | Blood | Male | March 2022 | 2096 |
| 81 | Urine | Female | March 2022 | 2096 |
| 82 | Blood | Male | March 2022 | 2096 |
| 84 | Urine | Female | March 2022 | 2096 |
| 85 | Urine | Female | March 2022 | 556 |
| 91 | Urine | Male | March 2022 | 348 |
| 92 | Urine | Female | March 2022 | 15 |
| 104 | Urine | Female | March 2022 | 3769 |
| 133 | Urine | Male | August 2021 | 1207 |
| 137 | Urine | Male | August 2021 | 1999 |
| 139 | Urine | Male | August 2021 | 1207 |
| 153 | Urine | Male | August 2021 | 4 |
| 165 | Urine | Female | September 2021 | 1801 |
| 167 | Urine | Male | September 2021 | 111 |
| 175 | Urine | Male | September 2021 | 661 |
| 181 | Urine | Female | September 2021 | 383 |

MLST: multi-locus sequence typing.

opposite [30,31]. Interestingly, an 8-year cross-sectional study from Jordan did not find differences in attitudes towards use of antimicrobials according to gender [32]. It would be difficult to explain our findings as we did not collect information on antimicrobials' use history or behavior from the individuals from which the isolates were obtained. It is possible that males could be misusing antimicrobials more than females. It might also be possible that societal differences would provide males with easier access, and hence higher chances of misuse of antimicrobials. This could be investigated in-depth in future studies.

Among the AME genes, the most frequent was *ant (3″)-I (*73.8 %), followed by *aac (6′)-Ib* (25.1 %), *aac (3′)-II* (17.5 %), *aph (3′)-VI* (12.0 %), *armA* (9.8 %), and *rmtB* (0.5 %). Among the PMQR genes, the most frequent was *oqxAB* (31.7 %), followed by *qnrS* (26.2 %), *qnrB* (25.7 %), and *aac(6′)-Ib–cr* (25.7 %). On the other hand, *qnrA, qnrD, qebA,* and *qnrC* were not detected in any of our isolates. The degree of agreement of the identified genes with reports from other countries varied from gene to gene and from country to country;

AMEs [10,11,13–16] and PMQR [11,18,19,33]. Several factors may contribute to observed differences in the non-susceptibility phenotypes and genotypes with those from other reports. These include differences in the rates of use and the misuse of antimicrobial agents, the clinical sources of the isolates, the socioeconomic characteristics of populations, differences in the prevalence of *K. pneumoniae* types, resistance genes and phenotypes from region to region, differences in study methodologies, the behaviors and attitudes toward use of antimicrobials, and the ease of access and availability to antimicrobials, among others.

In the present study, univariate analyses via the Pearson chi-squared test were used to identify possible associations among different variables. These included several genotypes and phenotypes. Based on these analyses, AME genes were significantly associated with non-susceptibility to aminoglycosides, which is expected. Studies from Iran, Turkey, and Egypt showed similar findings [10,11,15]. Similarly, PMQR genes were significantly associated with non-susceptibility to quinolones, which is expected. This was also reported by studies from Iran, Saudi Arabia, Taiwan, Korea, and Tunisia [19,34–37]. The presence of AME or PMQR genes among the isolates was significantly associated with the ESBL phenotype. This is likely related to the prevalence of ESBL genes among the isolates, which was not tested, as many resistance genes are spread together between isolates. Similar associations with the ESBL phenotype were reported by studies from Taiwan, Spain, Korea, Saudi Arabia, and Nigeria [12,16,37–39].

In the present study, many resistance genes were significantly co-associated with each other. Furthermore, the presence of a single type of resistance gene was significantly associated with many antimicrobial non-susceptible phenotypes. These findings indicate that many antimicrobial non-susceptibility phenotypes can be acquired at the same time because several resistance genes may be transferred concurrently, leading to an MDR phenotype. Indeed, 62.3 % of the isolates demonstrated the MDR phenotype. On the other hand, *qnrB* and *armA* showed statistically significant gene independence. According to studies from Korea and Taiwan a significant number of ESBL-producing *K. pneumoniae* isolates simultaneously harbored *armA* and *qnrB* [40,41]. While a study from China showed gene independence between the two [42]. This suggests that the degree of independence between *qnrB* and *armA* varies depending on the studied isolates and the studied populations.

Our study is the first to perform MLS analysis for *K. pneumoniae* from a wide range of infections and having a variable spectrum of antimicrobial susceptibility. Among the twenty-seven *K. pneumoniae* isolates analyzed using MLST, 19 sequence types were identified. ST2096 was the most frequent, and all isolates (n = 7) with this ST number were from the same hospital (data not shown). The detection of isolates with the same sequence type from one hospital may suggest the spread of this type within the hospital during the period of isolates' collection. Although the number of isolates subjected to MLST was modest, a preliminary conclusion of MLST analysis is that infections caused by *K. pneumoniae* were by strains having a wide range of genetic diversity.

**Table 9**
Susceptibility and ESBL phenotypes of identified isolates' sequence types, compared to previously reported phenotypes.

| This study's isolate IDs | Sequence type (ST) | Our findings | | | | Previously reported non-susceptible phenotype for the sequence type* |
|---|---|---|---|---|---|---|
| | | Aminoglycosides | Quinolones | Beta-lactams | ESBL phenotype | |
| 1 | 2648 | S | S | S | Negative | No report |
| 15 | 432 | S | S | S | Negative | ESBL positive |
| 30 | 348 | NS | S | NS | Positive | No report |
| 34 | 193 | S | NS | S | Negative | No report |
| 35 | 2096 | NS | NS | NS | Negative | No report |
| 43 | 2096 | NS | NS | NS | Negative | No report |
| 50 | 39 | NS | NS | NS | Positive | No report |
| 67 | 2343 | S | S | S | Negative | No report |
| 69 | 1198 | S | S | S | Negative | No report |
| 77 | 2299 | S | NS | S | Negative | No report |
| 78 | 2096 | NS | NS | NS | Negative | No report |
| 79 | 2096 | NS | NS | NS | Negative | No report |
| 81 | 2096 | NS | NS | NS | Negative | No report |
| 82 | 2096 | NS | NS | NS | Negative | No report |
| 84 | 2096 | S | S | S | Negative | No report |
| 85 | 556 | NS | NS | NS | Positive | No report |
| 91 | 348 | NS | NS | NS | Positive | No report |
| 92 | 15 | NS | NS | NS | Positive | To quinolones<br>To aminoglycosides<br>ESBL positive |
| 104 | 3769 | NS | NS | S | Negative | No report |
| 133 | 1207 | NS | NS | NS | Positive | No report |
| 137 | 1999 | S | S | NS | Positive | No report |
| 139 | 1207 | NS | NS | NS | Positive | No report |
| 153 | 4 | NS | NS | NS | Positive | No report |
| 165 | 1801 | NS | NS | NS | Positive | No report |
| 167 | 111 | NS | NS | NS | Negative | To carbapenems |
| 175 | 661 | NS | NS | NS | Negative | To quinolones |
| 181 | 383 | NS | NS | NS | Negative | No report |

S: susceptible. NS: non-susceptible. ESBL: extended spectrum beta-lactamase. * Previous reports on non-susceptibility of the sequence types were queried using the (pasteur.fr) field breakdown tool. "No report" indicates no previous data regarding susceptibility to aminoglycosides, quinolones, and beta-lactams, and the ESBL phenotype.

A previous study from Jordan used MLST and pRFLP to determine the genetic diversity of carbapenemase-producing *K. pneumoniae* clinical isolates from Amman, Jordan. In this study, 7 sequence types were identified among 17 *K. pneumoniae* isolates. The most frequent sequence type was ST147, followed by ST101. Among the identified sequence types, ST15 was common with our study [43]. Other studies from Jordan have investigated the level of genetic diversity among *A. baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA), and carbapenemase-producing *K. pneumoniae* [44,45]. Many molecular typing methods were used in these studies, including, pulsed-field gel electrophoresis, pRFLP, MLST, *spa* typing, and *agr* typing [44–46]. Like our findings, studies from the United Arab Emirates, Iran, China, Denmark, and Germany found ST15 among their *K. pneumoniae* isolates [47–51]. ST2096 was detected in Turkey [52], which is consistent with our findings. Our study is the first to report many non-susceptible phenotypes and the ESBL phenotype within certain STs of *K. pneumoniae* (per the (pasteur.fr) field breakdown tool), as indicated in Table 9.

Our study had several strengths. Many reliable methods and techniques were used, including species identification via the VITEK system, the Kirby-Bauer disk diffusion method for AST analysis, the double disk synergy test for ESBL phenotype identification, PCR for species identity confirmation and for genotypic detection of the studied resistance genes, MLST as a molecular typing method for selected isolates, and the use of DNA sequencing to confirm identity of the studied genes. The data were analyzed using Pearson's chi-squared test, providing reliable statistics to identify significant associations between resistance genotypes and phenotypes. Finally, isolates were from several major hospitals in northern Jordan that serve a significant percentage of the Jordanian population.

Our study had several limitations. A major limitation was the modest research budget allocation. The budget restriction led to an inability to collect and study more isolates, include more regions and hospitals, and investigate the prevalence of more resistance genes (e.g., ESBL genes). Many molecular typing methods combined with MLST, may be more effective at detecting the genetic diversity of isolates. However, including many typing methods is more expensive and thus was not considered. A larger sample size would have enabled obtaining more representative data, and potentially observing new associations. Many geographical regions of Jordan, bacterial species, and resistance genes can be included in future studies. This could help characterize the epidemiology of resistance genotypes and phenotypes among clinical isolates. Performing molecular typing of a larger number of isolates would enable the accurate characterization of prevalent isolates among the different Jordanian regions, as well as among the community and hospital settings.

## Ethics statement

Approval to conduct the study was granted by the IRB committee of Jordan University of Science and Technology (Approval ID 26/144/2021).

## Data availability statement

Data included in supplementary material.

## Ethics approval and consent to participate

This study was reviewed and approved by IRB committee of Jordan University of Science and Technology, with the approval number: 26/144/2021. Informed consent was not required for this study because of the retrospective nature of bacterial isolates collected.

## CRediT authorship contribution statement

**Samer Swedan:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Emad Addin Alabdallah:** Conceptualization, Data curation, Investigation, Writing - original draft. **Qutaiba Ababneh:** Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23368.

## Abbreviations

| | |
|---|---|
| AAC | aminoglycoside acetyltransferase |
| AME | aminoglycoside-modifying enzymes |
| ANT | aminoglycoside nucleotidyltransferases |
| APH | aminoglycoside phosphotransferase |
| ATCC | American type culture collection |
| CLSI | the clinical and laboratory standards institute |
| ESBL | extended-spectrum beta-lactamase |
| MDR | multiple drug resistant |
| MHA | Mueller-Hinton agar |
| MLST | multi-locus sequence typing |
| MRSA | methicillin-resistant Staphylococcus aureus |
| PCR | polymerase chain reaction |
| PMQR | plasmid-mediated quinolone resistance |
| pRFLP | plasmid restriction fragment length polymorphism |
| ST | sequence type |
| XDR | extensively drug resistant |

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