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Molecular characterization of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from King Abdulaziz Specialist Hospital at Taif City, Saudi Arabia

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ABSTRACT

Klebsiella pneumoniae is an opportunistic pathogen responsible for a significant proportion of nosocomial and community-acquired infections. Genotypic variation in *K. pneumoniae* populations is a major barrier to control public health risk associated with pathogen. In this work, thirty *K. pneumoniae* were recovered from hospital and were tested for their resistance to antibiotics. Genetic variability of the isolates was performed using PCR based on genes coding for porins and efflux pumps, $(GTG)_5$ and BOX repetitive sequences. *K. pneumoniae* showed heterogenicity of resistance to antibiotics based on gender or specimen type. Further, out of 30 isolates, 25 different profiles were found and 83.33% are multidrug-resistant. PCR detection of genes coding for porins and efflux pumps revealed seven different genotypes and strong correlation between antibiotics resistance profiles and investigated genes. PCR genomic fingerprinting showed high genetic diversity of *K. pneumoniae*. BOX-PCR and $(GTG)_5$ generated 18 and 19 clusters with discriminatory indexes 0.97 and 0.98, respectively at 80% of similarity. *K. pneumoniae* clinical isolates showed high phenotypic and genetic variability, and many strains can be circulating simultaneously. This genetic variability should be taken into consideration when designing strategies for controlling *K. pneumoniae* outbreaks. In addition, a significant correlation, was detected for the first time, between $(GTG)_5$-genotyping and antibiotic resistance patterns of *K. pneumoniae* and could be valuable in the prediction of antibiotic resistance profiles of *K. pneumoniae*.

Introduction

Klebsiella pneumoniae is a Gram-negative nosocomial pathogen that causes wide range of infections, such as pneumonias, urinary tract infections, bacteremias, and liver abscesses [1]. This opportunistic, encapsulated, nonmotile bacterium can survive in hospitals, persist on environmental surface and colonize human skin, respiratory tract and bowels [2]. *Klebsiella* can be transmitted via personal contact, contaminated environments and medical equipment [3].

Due to the high rates of resistance to antibiotics, *K. pneumoniae* was considered as one of the most frequent agents of infectious diseases and significant menace to public health [4]. The resistance to antibiotics is a complex multifactorial process [5]. Nevertheless, it reflects evolution in action, related to the continuous exposure to antibiotics. Consequently, the selective pressure gives rise to the development of many genetic mechanisms [6]. The acquired resistance over the years has led to the emergence of multidrug-resistant (MDR) and extensively

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drug-resistant (XDR) strains [7] that exhibit resistance to nearly all available antibiotics, without possible treatment options. The resistance of *K. pneumoniae* to many antibiotics is due to multiple mechanisms such as modification of antibiotic target sites, alteration of metabolic pathways, activation of efflux pump systems, change in the permeability of membrane and release of antibiotic-inactivating enzymes [8,9]. Among these mechanisms, the efflux pump systems and enzymatic degradation play a major role in the multidrug resistance development [10]. *K. pneumoniae* produces many enzymes, such as carbapenemases, metallo-β-lactamases, oxacillinases and extended-spectrum β-lactamases that can degrade β-lactam antibiotics. The efflux pumps, belonging to the resistance-nodulation-division (RND) family, can extrude aminoglycosides, fluoroquinolones and β-lactams [8,11]. Recently, the plasmid-mediated colistin resistance (MCR) genes were associated with the increase of colistin resistance *K. pneumoniae* [12].

Genotyping is a major tool for infection control to monitor the predominance of some strains within a healthcare institution or to investigate if a group of infections are related or not to outbreak [13]. Genetic diversity can now be performed by various methods to determine a phylogenetic relationship among bacteria such as *Klebsiella* and to generate epidemiological fingerprint profiles [14,15]. Repetitive extragenic palindromic-PCR based on BOXA1R and $(GTG)_5$ primers have been used widely for studying genetic relationship of different bacteria. Five rep-PCR genomic fingerprinting methods were tested and were found that $(GTG)_5$-PCR followed by BOX-PCR and are the most suitable methods for molecular typing of *E. coli* [16]. In addition, $(GTG)_5$-PCR has been considered as promising tool for genotyping of *K. pneumoniae* [17,18] and *Campylobacter concisus* [19].

This study aimed to identify the antibiotic resistance profiles of *K. pneumoniae* clinical isolates and the prevalence of porins and multidrug efflux pump genes. Furthermore, the genetic variability of the isolates and the relationship between genetic and phenotypic patterns were investigated.

Materials and methods

Bacterial isolates

Thirty *K. pneumoniae* isolates were recovered from patients at King Abdulaziz Specialist Hospital, Taif, Saudi Arabia. Strains were isolated from blood, sputum, urine, and wound swabs specimens. Isolates were identified by conventional and biochemical tests as described previously [20].

Antibiotic susceptibility

Antibiotic susceptibility of *K. pneumoniae* isolates was achieved using Vitek 2 system (bio-Mérieux, Inc., Durham, NC, USA) using software version 5.04 and the AST-GN69 and AST-XN06 cards [21,22], according to the manufacturer's instructions. The antibiotics tested were: Ampicillin (AMP), Piperacillin\Tazobactam (PRL), Cefoxitin (CX), Cefepime (FEP), Imipenem (IPM) 10 μg; Meropenem (MEM), Tetracycline (TE), Ciprofloxacin (CIP), Tigecycline (TGC), Nitrofurantion (NI), Trimethoprim-Sulfamethoxazole (SXT), Aztreonam (ATM), Piperacillin (PIP), Cefazolin (FAZ), Cefuroxime (CXM), Ertapenem (ETP), Fosfomycin (FOS), Levofloxacin (LE), Norfloxacin (NOR), Amikacin (AK), Gentamicin (CN), and Tobramycin (TOB). The Vitek 2 ESBL test is included on the AST-GN69 card. Strains that showed resistance to three or more different antimicrobials classes were defined as MDR [7].

Molecular characterization of K. pneumoniae *isolates*

DNA extraction

K. pneumoniae isolates were cultured in nutrient broth for 24 h at 37 °C. Bacteria were pelleted from 1.5 mL nutrient broth and suspended in 200 μL of sterile distilled water. The genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Detection by PCR of multidrug resistance genes

K. pneumoniae strains were screened for multidrug efflux pump system genes *acrAB*, *tolC* and *mdtK*, and genes coding for porins *ompK35* and *ompK36*.

PCR was carried out in 20 μL containing 50 ng of extracted DNA, 4 μL of GoTaq® Green Master Mix (Promega, Madison, WI, USA) and 1 μL of each forward and reverse respective primers (25 pM). PCR cycles conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, 30 s for primer annealing temperature (Table 1), and extension at 72 °C for 1.5 min; followed by a final extension at 72 °C for 10 min. PCR products were visualised on 1.5% agarose gel stained with ethidium bromide (0.5 mg/mL), photographed using Bio-Rad Gel Doc 2000 (Germany) and their sizes were determined with a 100 bp molecular size marker (Promega, Madison, WI, USA).

Genotyping of K. pneumoniae *isolates using* $(GTG)_5$ *and BOX methods*

Rep-PCR based on $(GTG)_5$ and BOXA1R-primers were used for genotyping *K. pneumoniae* isolates. PCR reaction was performed in a total volume of 25 μL including 12.5 μL of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 μL (10 pmol) of $(GTG)_5$ or BOXA1R primers (Table 1), and 50 ng of genomic DNA. PCR cycles conditions were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 2.5 min; followed by a final extension at 72 °C for 7 min. PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide (0.5 mg/mL), photographed using Bio-Rad Gel Doc 2000 (Germany) and their sizes were determined with a 100 bp molecular size marker (GeneDireX, Germany).

Cluster analysis

The banding patterns generated by BOX and $(GTG)_5$-PCR were displayed using PyElph version 1.4. The dendrograms were generated with Dice coefficient and the UPGMA clustering method (tolerance 1%), based on fingerprints of BOX-PCR and $(GTG)_5$-PCR.

Discriminatory index

Simpson's index of diversity [discriminatory index (D)], was calculated using to the following formula:

$$D = 1 - \frac{1}{N-1} \cdot \sum_{j=1}^{s} nj\,(nj-1)$$

where N is the total number of isolates in the sample population, s is the total number of types described, and *nj* is the number of strains belonging to the *j*th type. Simpsons index of diversity ranges from 0 to 1. A value of 1 is highly discriminatory, and a value of 0 is not discriminatory [25].

Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA). Spearman's correlation coefficient (r_s) and their significance (p) were calculated using SPSS 20.

Table 1
List of primers, expected amplicon size and annealing temperature.

Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Tm °C	Reference
acrAB	For: ATCAGCGGCCGGATTGGTAAA Rev: CGGGTTCGGGAAAATAGCGCG	312	53	
tolC	For: ATCAGCAACCCCGATCTGCGT Rev: CCGGTGACTTGACGCAGTCCT	527	51	
mdtK	For: GCGCTTAACTTCAGCTCA Rev: GATGATAAATCCACACCAGAA	453	43	[23]
ompK35	For: CTCCAGCTCTAACCGTAGCG Rev: GGTCTGTACGTAGCCGATGG	241	51	
ompK36	For: GAAATTTATAACAAAGACGGC Rev: GACGTTACGTCGTATACTACG	305	43	
$(GTG)_5$	GTGGTGGTGGTGGTG	–	52	[24]
BOXA1R	CTACGGCAAGGCGACGCTGACG	–	53	

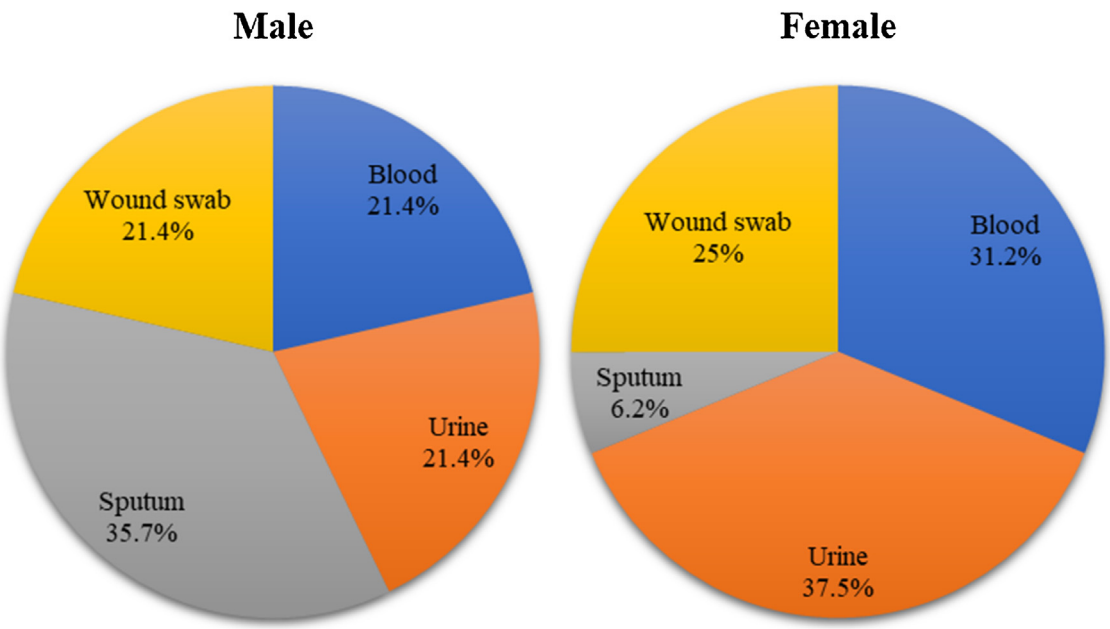

Fig. 1. Distribution of *K. pneumoniae* clinical isolates.

Results

Distribution of the isolates

This work was conducted on 30 isolates among them, 16 isolated from female patients (53.33%) and 14 from male patients (46.66%). Based on type of specimen, we have 26.7% isolates from blood, 30% from urine, 20% from sputum and 23.3% from wound swab. The distribution of isolates is presented in Fig. 1.

Antibiotic susceptibility testing

In total, 22 antibiotics were tested and isolates showed high variability of resistance. Firstly, all isolates were resistant to ampicillin (100%) as the highest resistance. The isolates also showed higher resistance to piperacillin (93.33%). The weakest resistance was observed in case of imipenem (13.33%) and 33% of the isolates were ESBL positive.

Based on gender (Fig. 2), the highest resistance rates of the 14 *K. pneumoniae* isolated from male patients was observed for ampicillin (100%) and piperacillin (85.71%). The weakest resistance was detected in cases of fosfomycin and tigecycline (21.42%) and 35.71% of the isolates were ESBL positive. In case of 16 female patients, we observed resistance to all tested antibiotics except imipenem. The highest resistance rates were noticed for ampicillin and piperacillin (100%). The weakest resistance was detected with piperacillin\tazobactam, meropenem, amikacin, ertapenem and tigecycline (12.5%). Furthermore, 31.25 % of the isolates appeared as ESBL positive. Student's *t*-test showed no significant difference of gender on antibiotics resistance, Male (8.32 ± 2.85), Female (6.64 ± 5.05) at *p*-value = 0.18. Chi-Square Test showed no statistically significant association between gender and ESBL production, χ = 0.067, p = 0.796.

Based on type of specimen (Fig. 3), it was noted that strains isolated from urine were resistant to all antibiotics except imipenem. The highest resistance rates were reported for ampicillin and piperacillin (100%), while the weakest resistance was detected in cases of piperacillin\tazobactam, meropenem, amikacin, ertapenem and tigecycline (22.22%). In addition, 33.33% of the isolates were ESBL positive. For blood isolates, a resistance to all antibiotics except imipenem was observed. The highest resistance rates were described for ampicillin, cefazolin, cefuroxime, piperacillin (100%), while the weakest resistance was detected with cefoxitin and nitrofurantoin (12.5%) and 25% of the isolates were ESBL positive. The sputum isolates were resistant to all antibiotics except fosfomycin. The highest resistance was encountered with ampicillin (100%). However, the weakest resistance was recorded in cases of imipenem and tigecycline (16.66%). Further, 66.66% of the isolates were ESBL positive. For the seven strains isolated from wound swab, a resistance to all antibiotics except

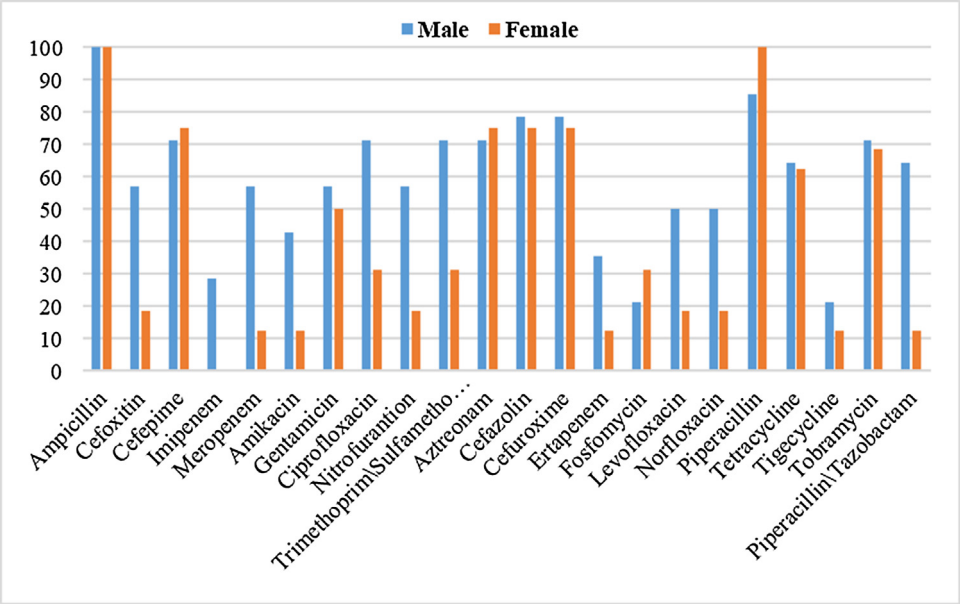

Fig. 2. Antibiotic resistance of *K. pneumoniae* clinical isolates based on gender.

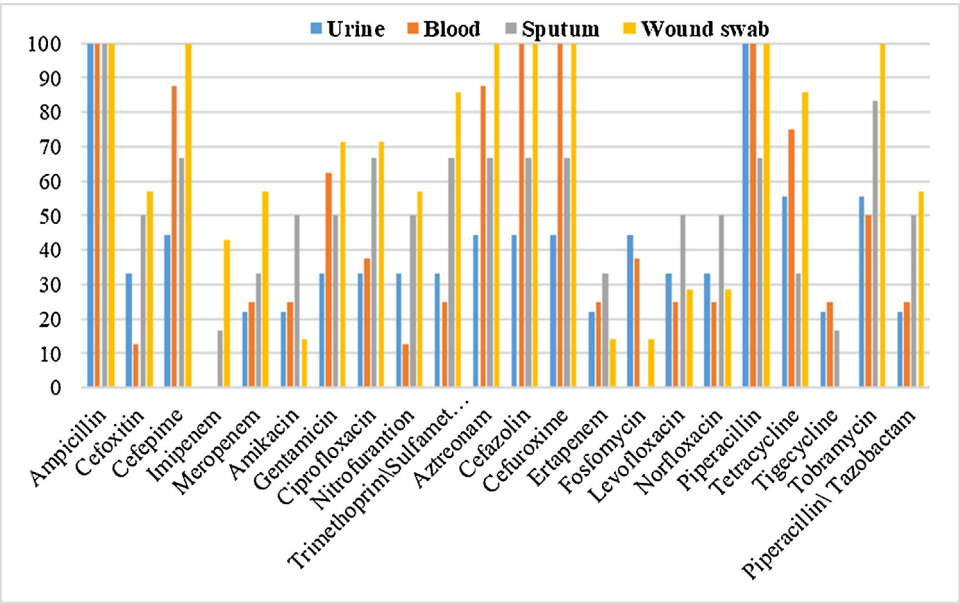

Fig. 3. Antibiotic resistance of *K. pneumoniae* clinical isolates based on type of specimen.

tigecycline was noted. The highest resistance was revealed for ampicillin, cefepime, aztreonam, cefazolin, cefuroxime, piperacillin and tobramycin (100%). Whereas, the weakest resistance was described with amikacin, ertapenem and fosfomycin (14.28%). In addition, 14.28% of the isolates were ESBL positive. Student's *t*-test showed no significant effect of specimen type on antibiotics resistance, urine (3.59 ± 2.08), blood (3.86 ± 2.71), sputum (3.09 ± 1.38), wound swab (4.40 ± 2.40) at *p*-value = 0.26. in addition, there was no statistically significant association between specimen type and ESBL production, $\chi = 0.493$, $p = 0.222$.

Out of the 30 isolates, we noted the existence of 25 profiles and 83.33% (25 isolates) were considered as MDR (Table 2). The highest resistance (20 antibiotics) was detected in the strains 8 and 15 isolated from male patients (sputum and blood respectively). In addition, strains 1 and 16 isolated from urine (male and female respectively) were resistant to 19 antibiotics. Further, the isolate 21 was resistant to 18 antibiotics, and the isolates 26 and 30 were resistant to 17 antibiotics. Several isolates presented the same profile (isolates numbers 8 and 15; 10 and 22; 13, 18 and 23; 28 and 29) although they are of different origin (gender and/or specimen type).

PCR Detection of porins and multidrug efflux pump system genes

PCR detection of genes coding for porins and multidrug efflux pump system revealed 7 different genetic profiles (Table 2). The *acrAB* gene coding for the multidrug efflux pump system was detected in 93.33% of the isolates. All strains isolated from female patients harbour this gene in contrary to 85.71% of male patients. In addition, all of urine and blood isolates harbour *acrAB* gene whereas it was detected in 85.71% and 83.33% of wound swab and sputum isolates respectively.

Table 2
Antibiotic resistance patterns of *K. pneumoniae* isolates and prevalence of genes coding for porins and efflux pumps.

Isolate	Specimen	Antimicrobial resistance profile	Genes coding for porins and efflux pumps					Genetic profile
			ompK35	*ompK36*	*mdtK*	*tolC*	*acrAB*	
1	Urine	AMP-PRL-CX-FEP-MEM-AK-CN-CIP-NI-SXT-ATM-FAZ-CXM-ETP-LE-NOR-PIP-TE-TOB	+	+	–	+	+	P1
2	Urine	AMP-NI-FOS-PIP	+	+	–	+	+	P1
3	Sputum	AMP-PRL-CX-FEP-AK-CN-CIP-NI-SXT-ATM-FAZ-CXM-LE-NOR-PIP-TOB	–	–	–	–	–	P2
4	Blood	AMP-FEP-ATM-FAZ-CXM-PIP-TE	–	+	–	+	+	P3
5	Urine	AMP-FOS-PIP	+	+	–	+	+	P1
6	Blood	AMP-PRL-MEM-AK-CN-CIP-SXT-FAZ-CXM-ETP-FOS-LE-NOR-PIP-TE-TGC	–	–	–	–	+	P4
7	Sputum	AMP-TOB	+	+	–	+	+	P1
8	Sputum	AMP-PRL-CX-FEP-MEM-AK-CN-CIP-NI-SXT-ATM-FAZ-CXM-ETP-LE-NOR-PIP-TE-TGC-TOB	+	+	–	+	+	P1
9	Blood	AMP-FEP-ATM-FAZ-CXM-FOS-PIP	–	+	–	–	+	P5
10	Urine	AMP-FEP-CIP-SXT-ATM-FAZ-CXM-LE-NOR-PIP-TE-TOB	+	+	–	+	+	P1
11	Urine	AMP-CX-FEP-CN-NI-ATM-FAZ-CXM-PIP-TE-TGC-TOB	+	+	–	+	+	P1
12	Blood	AMP-FEP-CN-CIP-ATM-FAZ-CXM-PIP-TOB	–	+	–	–	+	P5
13	Blood	AMP-FEP-CN-ATM-FAZ-CXM-PIP-TE-TOB	+	+	–	+	+	P1
14	Blood	AMP-FEP-ATM-FAZ-CXM-FOS-PIP-TE	+	+	–	+	+	P1
15	Blood	AMP-PRL-CX-FEP-MEM-AK-CN-CIP-NI-SXT-ATM-FAZ-CXM-ETP-LE-NOR-PIP-TE-TGC-TOB	+	+	–	+	+	P1
16	Urine	AMP-PRL-CX-FEP-MEM-AK-CN-CIP-SXT-ATM-FAZ-CXM-ETP-LE-NOR-PIP-TE-TGC-TOB	+	+	–	+	+	P1
17	Urine	AMP-PIP	+	+	–	+	+	P1
18	Blood	AMP-FEP-CN-ATM-FAZ-CXM-PIP-TE-TOB	–	+	–	+	+	P3
19	Sputum	AMP-FEP-AK-CN-CIP-SXT-ATM-FAZ-CXM-PIP-TE-TOB	+	+	–	+	+	P1
20	Urine	AMP-FOS-PIP-TE	+	+	–	+	+	P1
21	Wound swab	AMP-PRL-CX-FEP-MEM-CN-CIP-NI-SXT-ATM-FAZ-CXM-ETP-FOS-LE-NOR-PIP-TOB	+	+	–	+	+	P1
22	Wound swab	AMP-FEP-CIP-SXT-ATM-FAZ-CXM-LE-NOR-PIP-TE-TOB.	+	+	–	+	+	P1
23	Wound swab	AMP-FEP-CN-ATM-FAZ-CXM-PIP-TE-TOB.	+	+	–	+	+	P1
24	Sputum	AMP	+	+	–	+	+	P1
25	Urine	AMP-FOS-PIP-TOB	+	–	–	+	+	P5
26	Sputum	AMP-PRL-CX-FEP-PM-MEM-CIP-NI-SXT-ATM-FAZ-CXM-ETP-LE-NOR-PIP-TOB	+	+	+	+	+	P6
27	Wound swab	AMP-FEP-SXT-ATM-FAZ-CXM-PIP-TE-TOB	+	+	–	+	+	P1
28	Wound swab	AMP-PRL-CX-FEP-IPM-MEM-CN-CIP-NI-SXT-ATM-FAZ-CXM-PIP-TE-TOB	+	+	+	+	+	P6
29	Wound swab	AMP-PRL-CX-FEP-IPM-MEM-CN-CIP-NI-SXT-ATM-FAZ-CXM-PIP-TE-TOB	–	+	–	–	–	P7
30	Wound swab	AMP-PRL-CX-FEP-IPM-MEM-AK-CN-CIP-NI-SXT-ATM-FAZ-CXM-PIP-TE-TOB	+	+	+	+	+	P6

Ampicillin (AMP); Piperacillin\Tazobactam (PRL); Cefoxitin (CX); Cefepime (FEP); Imipenem (IPM); Meropenem (MEM); Amikacin (AK); Gentamicin (CN); Ciprofloxacin (CIP); Nitrofurantion (NI); Trimeth\Sulfa (SXT); Aztreonam (ATM); Cefazolin (FAZ); Cefuroxime (CXM); Ertapenem (ETP); Fosfomycin (FOS); Levofloxacin (LE); Norfloxacin (NOR); Piperacillin (PIP); Tetracycline (TE); Tigecycline (TGC); Tobramycin (TOB).

The *mdtK* gene encoding for the multidrug efflux pump system was found to be in only three *K. pneumoniae* strains isolated from male patients (10%) including two strains from wound swab and one from sputum. The *tolC* gene coding for transport channel was present in 83.33% of the isolates among them 93.75% from female patients and 71.42% from male patients. Furthermore, this gene was detected in 100% of urine and sputum isolates, and in 85.71% and 50% of wound swab and blood isolates respectively. The *ompK35* gene coding for porins was found to be in 76.66% of the isolates (81.25% of female and 71.42% of male patients). Based on specimen type, *ompK35* gene was identified in urine isolates (100%), wound swab isolates (85.71%) sputum isolates (83.33%) and blood isolates (37.5%). For *ompK36* gene, coding for the porins, it was identified in 90% of the isolates. *K. pneumoniae* strains isolated from female patients (93.75%) harbour this gene more than strains isolated from male patients (85.71%). Further, *ompK36* gene was detected in 100% of sputum and wound swab, in 88.88% of urine and in 75% blood isolates.

Spearman's rank-order correlation showed statistically significant strong positive correlations between antibiotic resistance patterns of the isolates and the presence of resistance genes as follow: *arcAB*, r_s = 0.991, p = 0.008; *tolC*, r_s = 0.985, p = 0.001; *mdtK*, r_s = 0.598; *ompK35*, r_s = 0.976, p = 0.001 and *ompK36*, r_s = 0.993, p = 0.001.

Molecular typing of K. pneumoniae *isolates using* $(GTG)_5$ *and BOX analyses*

Molecular typing of *K. pneumoniae* isolates using the $(GTG)_5$-PCR and BOXA-1R-PCR generated 4–11 and 5–17 bands, respectively, ranging from 200 to 2000 bp (Figs. 4 and 5). Clusters were obtained according to the arbitrary cutoff values 60%, 70% and 80% for grouping by genotype similarity. According to Table 3, $(GTG)_5$-PCR clustered the isolates into 4, 11 and 19 clusters, with discriminatory indexes of 0.58, 0.90 and 0.98, respectively. However, BOXA1R-PCR clustered the isolates into 3, 9 and 18 clusters, with discriminatory indexes of 0.43, 0.67 and 0.97 respectively.

Spearman's correlation coefficient showed that only $(GTG)_5$ genotyping significantly negative correlates with antibiotic resistance patterns (r_s = −0.385; p = 0.036). However, there is no

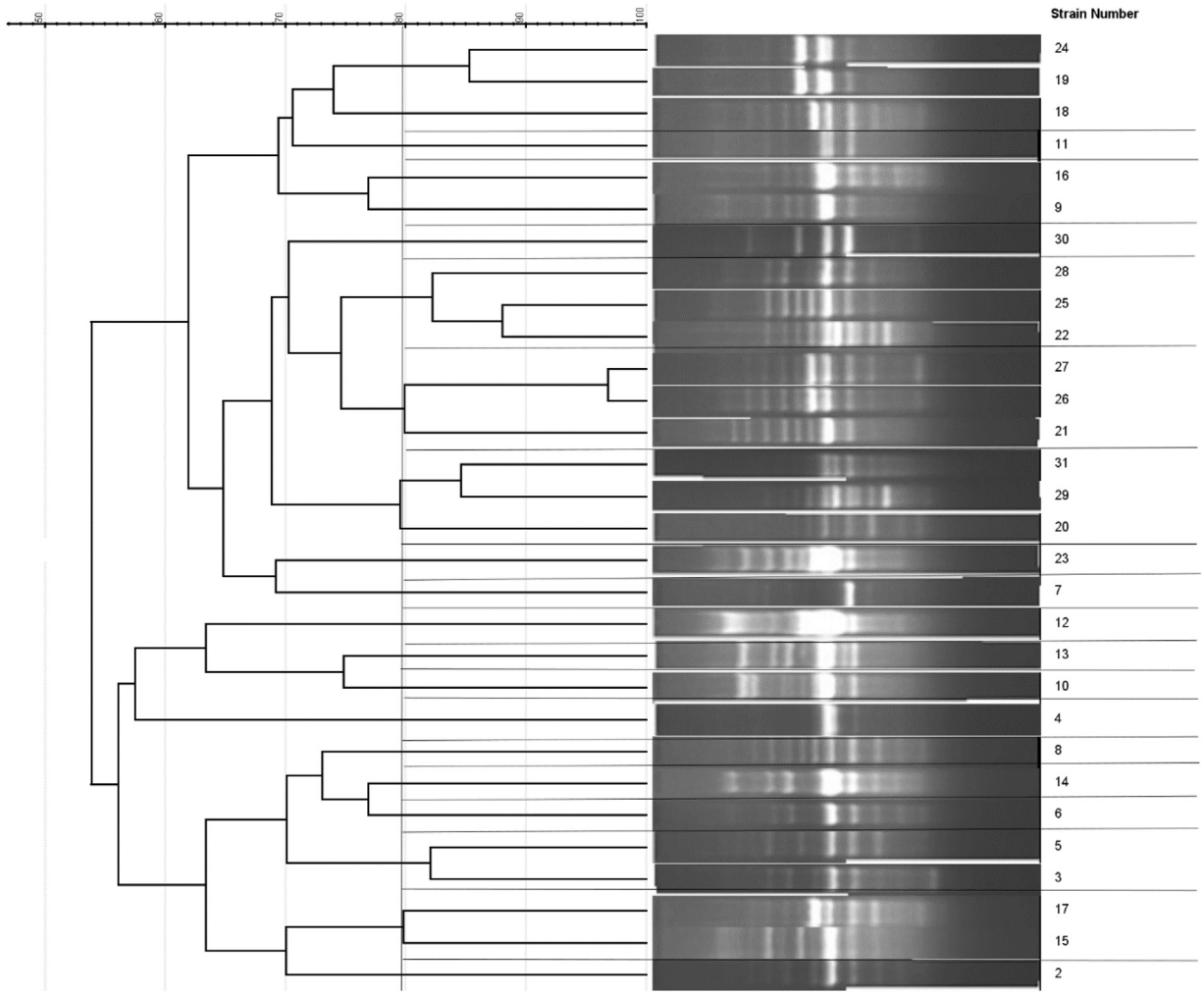

Fig. 4. Dendrogram generated with Dice coefficient and the UPGMA clustering method, showing the genetic similarity among *K. pneumoniae* clinical isolates by $(GTG)_5$-PCR genotyping.

significant correlation ($p > 0.05$) between genotyping methods and genes coding for porins and efflux pumps patterns of *K. pneumoniae*.

Discussion

K. pneumoniae is a common pathogen associated with both community and hospital-acquired infections including respiratory, urinary tract, wound and blood infections [26]. In this work 30 *K. pneumoniae* were isolated from blood, wound swab, urine and sputum of patients. The distribution of *K. pneumoniae* was slightly higher for female patients. This result was, on one side, in line with Kumarasamy et al. [27], who showed that the ratio of a female was about two to one of the isolates from Chennai and Haryana cities. On the other side the result was in disagreement with the studies of Osagieet al. [28], Akter et al. [29] and Nirwati et al. [30] who showed that *K. pneumoniae* infection was higher in males than in females. Based on specimen type, almost an equal distribution was distinguished in comparison to Seifi et al. [31] who reported that the majority of *K. pneumoniae* were isolated from urine.

In this work, *K. pneumoniae* isolates demonstrated high variability of resistance to antibiotics with 25 different profiles and 83.33% of the isolates were MDR. This high percentage of MDR is supported by previous studies [32,23], who showed that 90.2% and 71.1% respectively of the isolates were MDR. This high rate of resistance detected can be attributed to the overuse of antibiotics. The majority of investigated *K. pneumoniae* was resistant to diverse antibiotics, with ampicillin, piperacillin, cefuroxime, cefepime, cefazolin and aztreonam being the least effective for *K. pneumoniae* while tigecycline and imipenem had the most favorable profile. However, the least and the most favorable antibiotics varied slightly based on gender or specimen type despite that there is no significant effect of gender or specimen type on antibiotics resistance. This finding is in agreement with the work of Madahiah et al. [33] that found *K. pneumoniae* isolates were 100% resistant to ampicillin and is supported by previous studies [30,34] regarding ciprofloxacin and trimethoprim-sulfamethoxazole, in contrary to tobramycin and amikacin. The high resistance of *K. pneumoniae* isolates to beta-lactam indicates the production of special enzymes such as carbapenemase, and New Delhi Metallo-beta-lactamase [27], which provides resistance by deactivating the antibacterial properties of the antibiotics [35]. In addition to the cautious use of these antibiotics for the treatment of bacterial infection.

Various mechanisms are known to mediate antibiotic resistance to usually used antimicrobial agents, including carbapenemases, as well as 16S rRNA methyltransferase, aminoglycoside-modifying enzymes and ESBLs [36]. However, in this work, despite the high resistance of *K. pneumoniae* to tested antibiotics, only 33.33% of the isolates were ESBL positive. Thereby, another mechanism of resistance, such as genes coding for porins and efflux pumps, can be involved. Based on these genes, PCR revealed a genotypic variability

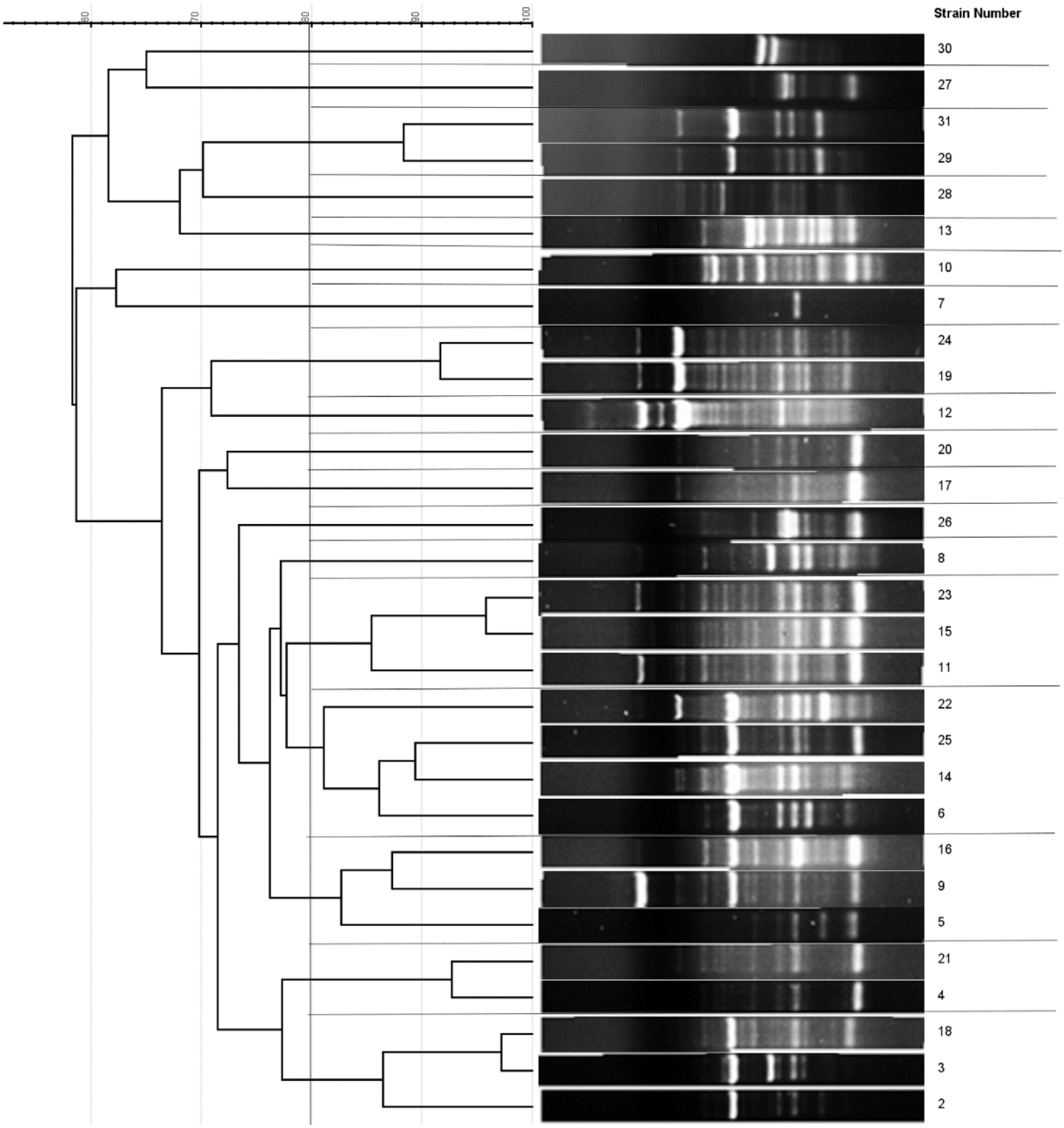

Fig. 5. Dendrogram generated with Dice coefficient and the UPGMA clustering method, showing the genetic similarity among *K. pneumoniae* clinical isolates by BOX-PCR genotyping.

Table 3
Discriminatory indices of $(GTG)_5$-PCR and BOX-PCR in genotyping of *K. pneumoniae*.

Discriminatory index	Cluster sizes	Number of clusters	Similarity %	Genotyping method
0.58	2, 3, 8, 18	4	60	
0.90	1, 1, 1, 1, 2, 2, 3, 3, 4, 5, 7	11	70	$(GTG)_5$-PCR
0.98	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 3, 3, 3	19	80	
0.43	2, 6, 22	3	60	
0.67	1, 1, 1, 1, 1, 2, 3, 3, 17	9	70	BOXA1R-PCR
0.97	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 3, 3, 4	18	80	

of the isolates with 7 different genetic profiles. Further, high prevalence of *acrAB* gene (coding for the multidrug efflux pump system) and *tolC* gene (coding for transport channel protein) was detected, which is in line with the report of Wasfi et al. [23]. Statistical analysis showed also significant strong positive correlation between antibiotics resistance patterns and the presence of resistance genes. Thereby, these genes may be implicated in the resistance of *K. pneumoniae* to antibiotics.

Antibiotic efflux pumps are among the major mechanisms of resistance to antimicrobials used by *K. pneumoniae* clinical isolates

[23]. It has been shown that the increased efflux of the antimicrobial agent leads to the reduction of its intracellular concentration, which can enhance bacterial survival [37]. In our work, a significant positive correlation was detected between the multidrug efflux pump genes (*acrAB-tolC*) and the antibiotic resistance patterns. Indeed, one of the principal efflux-related resistance mechanisms involves the expression of the *acrAB* gene, coding for a periplasmic protein (AcrA) and a transporter protein (AcrB) which use the TolC channel [38]. TolC acts as an outer-membrane channel that extrudes metabolites or antibiotics by cooperating with several inner-membrane or periplasmic proteins [39].

Outer membranes of gram negative bacterial are poorly permeable to hydrophilic and hydrophobic molecules. In this way, the majority of antimicrobial agents must cross the membrane in order to reach their intracellular drug targets and so require the presence of porin to bypass the membrane [40]. According to Kaczmarek et al. [41] the increase of resistance to carbapenem, ciprofloxacin, and chloramphenicol was related to the loss of porins ompK35 and ompK36. However, in this study, *ompK35* and *ompK36* porins were highly present and were significantly positive correlated to the antibiotic resistance patterns ($p < 0.05$). This may be attributed to the disruption in the protein coding sequence, presence of point mutations, or promoter region mutations in ompK35 and ompK36 porins [42].

Genotyping is a relevant tool for the nosocomial infections study. BOXA1R and $(GTG)_5$-PCR are the suitable method for bacterial discrimination [16]. In this work, out of the 30 *K. pneumoniae* isolates, $(GTG)_5$-PCR revealed 19 clusters with discriminatory index 0.98. However, BOXA1R-PCR generated 18 clusters, with discriminatory index 0.97 at 80% of similarity. This may be attributed to the genetic diversity of *K. pneumoniae* isolates. Our results are in agreement with other findings [17,23,43] that *K. pneumoniae* is highly heterogeneous, due to differences in nucleotide sequences. Based on Simpson's index of diversity $(GTG)_5$ was found to be slightly more suitable for *K. pneumoniae* discrimination than BOXA1R. In addition, the number of bands in $(GTG)_5$-PCR fingerprints is low than BOXA1R. The low number bands in fingerprints can be discriminative in small genomic variations and can be considered as advantage for $(GTG)_5$-PCR assay [17,44].

A Correlation between genotyping methods and profiles of antibiotic resistance was revealed by Wasfi et al. [23], Ashayeri-Panah et al. [45] and Espinar et al. [46] in *K. pneumonia*e. In this work, only $(GTG)_5$ genotypic analysis was found to be correlated, for the first time, with resistance patterns of *K. pneumoniae* indicating that may be valuable in prediction of *K. pneumoniae* resistance profiles. However, both $(GTG)_5$, and BOXA1R, did not show a significant correlation with genes coding for porins and efflux pumps patterns of *K. pneumoniae*.

Conclusions

According this finding, *K. pneumoniae* clinical isolates showed high heterogenicity of resistance to antibiotics. PCR genomic fingerprinting based on $(GTG)_5$ and BOX repetitive sequences and PCR detection of genes coding for porins and efflux pumps revealed high genetic diversity of the isolates. Thus, these isolates can be circulating simultaneously. Genetic variation of *K. pneumoniae* is an important barrier to control public health risk associated with pathogen thereby this diversity should be taken into consideration when designing strategies for controlling *K. pneumoniae* outbreaks. Correlation, detected for the first time, between $(GTG)_5$-genotyping and antibiotic resistance patterns of *K. pneumoniae* could be valuable in prediction of resistance patterns of *K. pneumoniae*.

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Competing interests

None declared.

Ethical approval

Not required.

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