Epidemiological typing of multidrug-resistant *Klebsiella pneumoniae*, which causes paediatric ventilator-associated pneumonia in Egypt

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Abstract

**Purpose.** Multidrug-resistant *Klebsiella pneumoniae* is a common nosocomial pathogen that plays an important role in ventilator-associated pneumonia (VAP). This study aimed to define the clonal relatedness of *K. pneumoniae* strains isolated from paediatric VAP in addition to those isolated from environmental samples.

**Methodology.** This study included 19 clinical and 4 environmental *K. pneumoniae* isolates recovered from the paediatric intensive care unit (PICU) in Assiut University Children’s Hospital. The *K. pneumoniae* isolates were confirmed by biotyping using API strips and subjected to antimicrobial susceptibility testing. The genes coding K1 and K2 capsular types were detected by PCR. The clonal relationships between the *K. pneumoniae* isolates were determined by pulsed-field gel electrophoresis (PFGE).

**Results.** Ten resistotypes were detected among all the *K. pneumoniae* isolates, while PFGE identified seventeen *K. pneumoniae* pulsotypes. Similar PFGE patterns were found between environmental and clinical isolates and between isolates recovered from different patients, suggesting the circulation of *K. pneumoniae* pathogens in the PICU and the role of the environment in the spread of infection. No correlation was found between the resistotypes and pulsotypes of the *K. pneumoniae* isolates. PFGE showed higher discriminatory power for the typing of nosocomial *K. pneumoniae* [Simpson’s diversity index (DI)=0.96] than resistotyping (DI=0.72).

**Conclusion.** As far as we know, this is the first report of the isolation of the same multidrug-resistant (MDR) *K. pneumoniae* pulsotype from patients and environmental samples in the same hospital ward in Egypt. This study provides a step on the way to understanding the genotyping and epidemiology of MDR *K. pneumoniae* for enhanced prevention of bacterial transmission.

INTRODUCTION

Ventilator-associated pneumonia (VAP) is a growing health-care-associated infection that causes significant morbidity and mortality, especially in developing countries [1, 2]. The incidence of VAP is higher in the intensive care units (ICUs) of Egyptian university hospitals than in those of other countries [3]. *Klebsiella pneumoniae* is a common bacterial pathogen responsible for a high percentage of nosocomial infections at paediatric units [4, 5], and was found to be the most common causative organism of VAP in Egypt [6, 7]. The empirical use of antibiotics and persistent exposure to a wide range of antimicrobials has led to the increased prevalence of multidrug-resistant (MDR) *K. pneumoniae* [8, 9].

Although there are many capsular serotypes of *K. pneumoniae*, the K1 and K2 types make up the majority of the virulent strains of *K. pneumoniae*. They have the ability to cause life-threatening invasive infections in young individuals [10–12]. No study concerning the prevalence of these serotypes in nosocomial *K. pneumoniae* infection in Egypt is available.
**K. pneumoniae** was found to be easily transferred through the hospital environment [13, 14]. Localizing the source of infection is the best approach to eliminate such spreads and transfers of this organism. Rapid and discriminative subtyping methods are useful for determining the clonal relatedness of nosocomial **K. pneumoniae** infections and hence tracing the source of infection [15, 16]. The commonly applied typing methods for **K. pneumoniae** are biotyping [17], phage typing [18], serological typing [19], antimicrobial sensitivity profiles (resistotyping) [20] and molecular typing. Molecular typing methods include multilocus sequence typing (MLST), random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) [21–24]. Despite the widespread distribution of **K. pneumoniae** in Egyptian VAP patients, the clonal similarity of these isolates had not yet been determined. The aim of this study was to describe the clonal relationship of MDR **K. pneumoniae** strains isolated from paediatric VAP as well as from paediatric intensive care unit (PICU) environmental samples.

**METHODS**

**Bacterial isolates**

This study was carried out on 23 **K. pneumoniae** strains isolated from the PICU at Assiut University Children’s Hospital from May 2014 to May 2015. These isolates included 19 clinical **K. pneumoniae** pathogens recovered from the endotracheal aspirates of 51 VAP-infected patients admitted to the PICU, as well as 4 strains isolated from 100 environmental samples (1 isolate was obtained from the floor, 2 were obtained from the tubes of ventilator circuits and 1 was obtained from a side table in the PICU).

VAP was diagnosed according to the Centers for Disease Control/National Healthcare Safety Network criteria for the diagnosis of paediatric VAP, including clinical, radiologic and bacteriologic evidence of infection, using quantitative endotracheal aspirate (EA) culture [25]. **K. pneumoniae** isolates were confirmed by conventional biochemical tests and API20E strips (bioMérieux) according to the manufacturer’s instructions.

**Antibiotic susceptibility testing**

The antimicrobial susceptibility testing for the isolated **K. pneumoniae** was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar according to Clinical and Laboratory Standards Institute guidelines [26]. The following commercial antimicrobial discs were used. Penicillin derivatives: piperacillin (100 µg); β-lactamase inhibitor combination: amoxacillin–clavulanic acid (20, 10 µg); aminoglycosides: amikacin (30 µg) and gentamicin (10 µg); carbapenems: imipenem (10 µg) and meropenem (10 µg); cephalosporins: cefazolin (30 µg), cefoperazone (75 µg), ceftriaxone (30 µg), cefepime (30 µg) and ceftazidime (30 µg); fluoroquinolones: levofloxacin (5 µg); and folate pathway inhibitor: trimethoprim/sulphamethoxazole (1.25/23.75 µg). *E. coli* ATCC 25922 strain was used for quality control. Isolates that showed resistance to at least three classes of antibiotics were considered as MDR strains, whereas the resistotype of each **K. pneumoniae** isolate was based on its resistance pattern to the seven tested antimicrobial classes.

**Molecular detection of capsular types K1 and K2**

DNA was extracted from all of the isolates by the boiling lysis method. The presence of *wzy_K1* and *wzy_K2* (K1/K2 capsule type) genes was tested using PCR. The primers used for the amplification of the *wzy_K1* gene were *wzy_K1*-F (5′-GGTGCTCTTTACATCATTGC-3′) and *wzy_K1*-R (5′-GCAAAGCCATT GGCTTAG-3′). The primers used for the amplification of the *wzy_K2* gene were *wzy_K2*-F (5′-GGATTATGACAGGCTCCTGCT-3′) and *wzy_K2*-R (5′-CGACTTGGTCACCAAGTT-3′) [27].

**PFGE**

PFGE was performed for all of the tested *K. pneumoniae* isolates according to the PulseNet protocol of the Centers for Disease Control [28]. Genomic DNA was macro-restricted with 50 U of *XbaI* restriction endonuclease and electrophoresis was performed on 1% certified Megabase agarose in a CHEF-DR III system (Bio-Rad). The following conditions were selected. Initial switch time: 6.7 s; final switch time: 35.3 s; voltage: 6 V cm⁻¹; included angle: 120°; and run time: 22 h at 14 °C. After PFGE, the gels were stained with ethidium bromide and then scanned under UV light. The band profiles were analysed using the GelCompar II software package (Applied Maths, version 6.6) and the relatedness was calculated using the criteria of Tenover et al. [29].

**Simpson’s diversity index (DI)**

Measurement of the genetic diversity and discriminatory power of the typing techniques was based on Hunter and Gaston’s formula [30].

**RESULTS**

The clinical *K. pneumoniae* isolates exhibited the MDR phenotype and were resistant to piperacillin, amoxacillin/clavulanate, cefazolin, cefepime, cefoperazone, ceftazidime and ceftriaxone. The environmental *K. pneumoniae* strains showed different sensitivity patterns to antimicrobials [two were MDR, one was resistant to two drug classes and one was single-drug resistant (SDR)]; however, surprisingly, all strains were resistant to meropenem. Ten resistotypes were detected among the total clinical and environmental *K. pneumoniae* isolates (Table 1).

A total of 23 clinical and environmental *K. pneumoniae* were tested by PCR for the detection of *wzy_K1* and *wzy_K2* genes. Only three clinical *K. pneumoniae* isolates tested positive for the *wzy_K2* gene, while all the isolates were negative for the *wzy_K1* gene (Fig. 1).

All tested *K. pneumoniae* isolates were genotyped by PFGE, which showed that the isolates had great genetic diversity (Fig. 2). From the dendrogram (Fig. 3), isolates sharing
Table 1. Resistotyping of clinical and environmental K. pneumoniae isolates

<table>
<thead>
<tr>
<th>Resistotyping code</th>
<th>Antimicrobial classes to which the isolates are resistant†</th>
<th>Isolates‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>(1, 2, 3, 4, 5, 6, 7)</td>
<td>K4, K6, K7</td>
</tr>
<tr>
<td>R2</td>
<td>(1, 2, 3, 4, 5, 7)</td>
<td>K1, K2, K3, K8, K10, K11, K13, K14, K15, K16, K17, K18</td>
</tr>
<tr>
<td>R3</td>
<td>(1, 2, 3, 4, 5, 6)</td>
<td>K12</td>
</tr>
<tr>
<td>R4</td>
<td>(2, 3, 4, 5, 6, 7)</td>
<td>KE2</td>
</tr>
<tr>
<td>R5</td>
<td>(1, 2, 3, 4, 6, 7)</td>
<td>K5</td>
</tr>
<tr>
<td>R6</td>
<td>(1, 2, 3, 4, 7)</td>
<td>K19</td>
</tr>
<tr>
<td>R7</td>
<td>(1, 2, 3, 7)</td>
<td>K9</td>
</tr>
<tr>
<td>R8</td>
<td>(2, 3, 4)</td>
<td>KE3</td>
</tr>
<tr>
<td>R9</td>
<td>(3, 4)</td>
<td>KE4</td>
</tr>
<tr>
<td>R10</td>
<td>(4)</td>
<td>KE1</td>
</tr>
</tbody>
</table>

†(1) Penicillin derivatives; (2) β-lactamase inhibitor; (3) cephalosporins; (4) carbapenem; (5) aminoglycosides; (6) fluoroquinolones; (7) folate pathway inhibitor.
‡K, clinical K. pneumoniae; KE, environmental K. pneumoniae.

more than or equal to 80% of the bands were classified in the same clusters. PFGE identified 17 pulsotypes of K. pneumoniae among 23 isolates and these were designated as pulsotypes A to Q. Pulsotypes A (n=4), B (n=2) and C (n=3) had >80% similar PFGE profiles among the isolates of each cluster. The remaining isolates (n=14) were not clonally related, i.e. they exhibited <80% similar PFGE profiles.

The Simpson’s diversity index (DI) for PFGE was 0.96, while for resistotyping it was 0.72.

**DISCUSSION**

*K. pneumoniae* is known as one of the significant causative agents of paediatric VAP worldwide [31–35]. In Egypt, *K. pneumoniae* is considered to be the commonest isolated microorganism for paediatric and neonatal VAP infection [6, 7, 34]. Limiting the hospital spread of *K. pneumoniae* strains requires the use of rapid and accurate epidemiological typing of the isolated strains [15, 16]. In this study, we investigated the clonal relatedness of *K. pneumoniae* strains isolated from VAP paediatric patients admitted to the PICU at Assiut University Children’s Hospital, Egypt. We used antimicrobial sensitivity profiles as the phenotyping method and PFGE pulsotyping as the genotyping method for epidemiological typing of isolated *K. pneumoniae* strains.

Our results showed that high-level MDR existed among all the clinical isolates against different classes of antibiotics. Two of the environmental *K. pneumoniae* were also found to exhibit MDR phenotypes. These results suggest that PICUs represent a well-established reservoir for MDR *K. pneumoniae* due to the misuse of antibiotics among paediatric patients. Three clinical *K. pneumoniae* isolates were positive for the *wzy*K2 gene, while all of the isolates were negative for the *wzy*K1 gene. This is the first report, to our knowledge, of K2-capsular-type *K. pneumoniae* strains in Egypt. These findings are in agreement with previous studies that reported that K1 and K2 *K. pneumoniae* occur more frequently in isolates recovered from community-acquired infections than those from hospital-acquired infections and that K2 is mostly found in respiratory-tract specimens [11, 35, 36].

Two typing methods were used in this study to detect the epidemiology of *K. pneumoniae* isolates from the PICU in Assiut University Hospital: resistotyping and PFGE typing. In our study, *K. pneumoniae* isolates were classified into resistotypes according to their resistance pattern to seven different antimicrobial classes. The most commonly found resistotype was R2, which exhibited resistance to 6 different antimicrobial classes (all classes except fluoroquinolones) and this resistotype included 12 isolates. PFGE identified 17 pulsotypes in clinical and environmental *K. pneumoniae* isolates, which were designated as pulsotypes A to Q. On analysing the association between the resistotypes and pulsotypes of *K. pneumoniae* isolates, it was found that strains with a similar resistotype usually belonged to multiple PFGE pulsotypes [16, 20]. The three identical *K. pneumoniae* strains belonging to pulsotype C had three different resistotypes (R6, R8 and R10). On the other hand, isolates K10 and K14 had the same pulsotype (B) and the same resistotype (R2) (Table 2). This difference might be explained by the fact that chromosomally identical *K. pneumoniae* strains had various antimicrobial sensitivity profiles; this usually results from the presence of plasmid-mediated resistance genes [16, 37].

It is prudent to say that *K. pneumoniae* typing on the basis of drug sensitivity profiles used to be one of the most frequently used methods in hospital epidemic [20]. Recently, it was suggested that antibiotic susceptibility testing has relatively limited utility as a typing system in epidemiological studies because antibiotic resistance is affected by surprising selective pressure in hospitals [15, 38]. In this study, resistotyping alone was not sufficient for discrimination between *K. pneumoniae* isolates and it needed to be powered by

![Fig. 1. Gel electrophoresis of the PCR-amplified products for detection of the wzy_K2 gene. Lane (M): DNA marker (100–3000); lane (1): negative control; and lanes (2), (3) and (4): positive for the wzy_K2 gene (1200 bp).](image-url)

**Fig. 3.** Dendrogram representing the PFGE profiles of 23 clinical and environmental *K. pneumoniae* isolates.
In this study, PFGE typing showed that three clinical K. pneumoniae isolates and one environmental isolate belonged to the same PFGE cluster (pulsotype A) and two environmental isolates and a clinical isolate belonged to the same pulsotype (C). Previous studies reported similar PFGE results for K. pneumoniae recovered from patients and environmental samples, which suggests that they have been involved in the spread of infection [39, 41]. Interestingly, one of the clinical K. pneumoniae isolates belonging to pulsotype B was obtained at the beginning of the study, while the other was taken 4 months later. The isolation of the same K. pneumoniae clone from different patients and environmental samples over this period of time indicates the circulation of these strains in the PICU. However, the clonal diversity among other isolates suggested that some strains could not be maintained or spread among paediatric patients [42].

The accumulation of resistance to different antimicrobials suggested the sequence of the transmission of K. pneumoniae isolates and the role of the environment in the spread of infection in the PICU. For isolates belonging to pulsotype A we could presume that the infection was started by a KE4 K. pneumoniae strain isolated from the environment (the side table in the PICU), and that this then moved to three patients, where it kept accumulating resistance, resulting in K8, K11 and K12, which belong to pulsotype A but express resistance to more drug classes. For isolates belonging to pulsotype C, it could be proposed that the K. pneumoniae moved from the environment (KE1 or KE3) to a patient (K19) that expressed an additional drug resistance phenotype. Our results supported what had previously been reported about the role of the environment in the transfer of infectious agents [43, 44].

Understanding the central mechanisms that feature in MDR K. pneumoniae infections, including tracing the source of infection and the clonal relationship between isolated strains, is the cornerstone for the development of efficient treatment policies as well as appropriate infection control measures. Further studies are required for the PFGE typing of K. pneumoniae isolated from other wards of Assiut University Children’s Hospital to estimate the genetic relatedness and locate the sources of infection of this pathogen. To our best of knowledge this is the first study in Egypt to describe the clonal relatedness of K. pneumoniae strains isolated from paediatric VAP patients.

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Conflicts of interest
The authors declare no conflicts of interest.

Ethical statement
The Ethics Committee of the Faculty of Medicine, Assiut University approved this study according to the latest revision of the Declaration of Helsinki, and informed consent was obtained from the participants’ parents or legal guardians.

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