AsPac J. Mol. Biol. Biotechnol. 2024
Vol. 32 (4) : 151-159

Molecular typing of multidrug resistant *Klebsiella pneumoniae* recovered from Iraqi burned patients

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Received 11th June 2024 / Accepted 11th November 2024 / Published 31st December 2024

Abstract. *Klebsiella pneumoniae* causes lethal nosocomial infections, mostly affecting patients with severe burns. More than 80% of its isolates have shown resistance to routinely used antibiotics in parallel with increased infection rates. The study aimed to determine the molecular typing and genetic relatedness of *K. pneumoniae*. Therefore, 20 multidrug resistant (MDR) *K. pneumoniae* already isolated from infected burned wounds in two major hospitals of Al-Kut city east Iraq were subjected to genotyping analysis. The random amplified polymorphic DNA (RAPD)-based polymerase chain reaction (PCR) technique was used along with three oligonucleotide primers (P13, OPX-04, and OPY-01). The amplicons' patterns of the electrophoresis-gel were analyzed by the GelJ software. Results revealed various patterns of DNA bands. A genetic similarity was seen within isolates from some locations. This genetic relatedness was captured by dendrogram analysis of the generated RAPD profiles. However, a genetic diversity among *K. pneumoniae* clinical isolates was also detected suggesting their different origins as well as ongoing changes of the bacterial genome. Furthermore, this could propose the circulation of many strains simultaneously within the hospitals. Therefore, it is important to consider this genetic heterogeneity when developing control measures for nosocomial *K. pneumoniae* infections. In conclusion, the current study highlights the dissemination of various MDR *K. pneumoniae* strains in the burn wards of two major hospitals in Al-Kut city, Iraq. Similar studies need to be performed in other Iraqi hospitals to establish a data base used in infection control systems, to predict, and manage the spread/outbreaks correlated with certain genotypes of resistant strains.

Keywords: burn wound infections, genetic diversity, *Klebsiella pneumoniae,* molecular typing, RAPD-PCR

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen responsible for numerous nosocomial and community-acquired infections (Lagha *et al.,* 2021). This bacterium has several virulence features, such as siderophores, capsular polysaccharides, type 1 and 3 fimbriae, and aggregative adhesion that allow it to cause significant illness and mortality (Podschun & Ullmann, 1998; Vuotto *et al.,* 2014). *Klebsiella* species may thrive on environmental surfaces, in hospitals, and inhabit the respiratory tract, stomach, and skin of people (Boll *et al.,* 2012). These pathogens can spread through physical contact, contaminated medical equipment, and surroundings (Hou *et al.,* 2015).

Strains of *K. pneumoniae* isolated from burn wound infections have been found to be more virulent than those grown from other infections (Aljanaby & Alhasnawi, 2017). Significant frequencies of multi-drug resistant (MDR) and extended spectrum β-lactamase (ESBL)-producing *K. pneumoniae* isolates have been identified in Iraq, particularly from burnt individuals (Aljanaby & Alhasani, 2016; Aljanaby & Alhasnawi, 2017; Ronat *et al.,* 2014). This necessitated the use of epidemiological methods to manage infection in hospital environments by monitoring the local spread of resistant bacterial

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strains (Johnson, 2015). Given the expansion of MDR *K. pneumoniae* in Iraqi hospitals, studying the isolates' molecular type and genotypic homology might provide a better knowledge of bacterial transmission (Akya *et al.,* 2018), as well as bacterial sources or origins (Akya *et al.,* 2018; Azimi *et al.,* 2019). Furthermore, genotyping is a significant tool for surveillance and control infections in healthcare institutions, as well as determining whether the illness is related with an epidemic (Lagha *et al.,* 2021).

Various genotyping approaches are available to identify the phylogenetic connection between *Klebsiella* species isolates and develop profiles of epidemiological fingerprints (Brisse *et al.,* 2001; Sardan *et al.,* 2004). One of these approaches is random amplified polymorphic DNA (RAPD)-based PCR, which has been widely utilized in epidemiological research for typing diverse species since it is simple and quick (Neela *et al.,* 2005). Further, it has been employed in population genetics, molecular ecology, and microbiology because it provides genetic markers at the genus, species, and strain levels (Argenton *et al.,* 1996). The main advantages of the RAPD method are its speed, its adaptability to any organism (as it does not require knowledge of nucleotide sequence, cell cycle, or chromosome complement), and its sensitivity in detecting a wide range of DNA damage and mutations (Lee *et al.,* 2007; Liu *et al.,* 2008; Noel & Rath, 2006). It has been employed for species and strain identification and genetic diversity studies (Hasan & Lafta, 2021; Hasan *et al.,* 2023; Mraidi & Lafta, 2021), genetic marker-assisted breeding (Liu *et al.,* 1999), and genotoxicity testing of environmental contaminants (Rocco *et al.,* 2011a, Rocco *et al.,* 2012b).

In this method, a short and single oligonucleotide primer of 9-10 bases is utilized to amplify random sequences inside a genomic DNA template by binding to various loci (Kumar & Gurusubramanian, 2011). Compared to other methodologies, RAPD is an alternative technique that has the potential of discovering polymorphisms throughout the genome (Gurakan *et al.,* 2008). Moreover, the afore-mentioned authors indicated that sequence changes in one or two primer binding sites cause polymorphisms due to the presence or lack of certain RAPD bands. Given the rise of MDR strains of *K. pneumoniae* in Iraqi hospitals, this study aimed to perform the molecular typing of *K. pneumoniae* clinical isolates, as well as explore the genetic relatedness among the isolates circulating in two hospitals in Al-Kut city, Iraq.

MATERIALS AND METHODS

Ethical approval

The samples utilized in this investigation were taken from a previous study done by Sadeq & Lafta (2024), who were authorized to collect burn wound samples from patients after getting an ethical permission no. 486 on 13th March 2022 granted by Wasit Health Directorate, Ministry of Health, Iraq.

Bacterial isolates

Twenty *K. pneumoniae* isolates were obtained from the study of Sadeq & Lafta, (2024). These isolates were recovered from dozens of burn wound swabs collected from patients hospitalized to two hospitals in Al-Kut city, Wasit governorate, Iraq. Isolates 1–5 were taken from patients hospitalized to Al-Karama hospital, whereas isolates 6–20 were collected from Al-Zahraa hospital patients. The isolates were identified as *K. pneumoniae* by polymerase chain reaction (PCR) and tested for antibiotic resistance using the Vitek2 assay (Sadeq & Lafta, 2024).

DNA extraction

To extract DNA, *K. pneumoniae* isolates were grown in nutrient broth (Tmmedia, India) overnight at 37°C. The genomic DNA was isolated from the bacterial growth using the EasyPure® Bacterial Genomic DNA Extraction Kit procedure (Transgenbiotech, China).

Genotyping by using RAPD-PCR

The octamer primers utilized in this investigation are available commercially as P13, OPX-04, and OPY-01 (Macrogen, Korea). They were chosen based on the presence of their sequences in many sections of the genomes of several strains of *K. pneumoniae* using the 'Sort sequence locator' from the free online program '*In silico* simulation of molecular biology experiments' (http://insilico.ehu.es/). The PCR amplification cycles

were optimized according to Hasan and Lafta's (2021) study to determine the optimal PCR annealing temperature. Then, the PCR reaction mixture was prepared, which consisted of 12.5 µl of AccuPower PCR Premix (Bioneer, USA), 2 µl of genomic DNA template (30 ng/µl), 1 µl of an octamer primer (20 pmol) whose sequences are depicted in Table 1, and up to 20 µl of deionized distilled water (ddH_2O). The RAPD-PCR procedure consisted of initial denaturation at 95°C for 5 min, followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 1 min, and then one cycle of final extension at 72°C for 7 min. After running the Prime Thermal Cycler (Cole-Parmer, UK), the PCR amplicons were observed on a 1.5% agarose gel stained with 0.5 mg/ml ethidium bromide, and the electrophoresis was performed at 70 v/mAmp for 1 h. The separated DNA bands were seen using a UV transilluminator (Biobase, China), photographed using a Gel imaging system (Germany), and their sizes were measured by comparing to a 100 bp molecular size marker (GeneDireX, Germany).

In silico analysis

The RAPD-PCR fingerprint pictures were analyzed and compared using the GelJ version2.0 software on a MacBook computer. This software was upgraded and updated by Heras *et al.* (2015).

RESULTS

RAPD-PCR based on the P13 primer

Using the P13 octamer primer, the random amplified polymorphic DNA-based polymerase chain reaction (RAPD-PCR) produced a variety of banding patterns on an agarose gel (Figure 1). The aforementioned figure clearly shows that *K. pneumoniae* isolates obtained from Al-Karama hospital (isolates 1–5) differed from those isolated from Al-Zahraa hospital. When the Al-Karama hospital isolates were compared to one another, isolates 3 and 4 were compatible since they did not display any amplified bands. In the case of the Al-Zahraa hospital isolates, despite the presence of several comparable bands, there are a few missing or extra bands in the gDNA. This discrepancy in bands patterns was confirmed by examining these bands using the GelJ program. Based on the constructed dendrogram in Figure 1, the isolates were categorized into five clusters with 70% similarity, particularly the bacteria isolated from Al-Zahraa hospital, indicating genetic relatedness.

RAPD-PCR based on the OPX-04 primer

Using the OPX-04 primer, the RAPD-PCR assay was able to amplify many bands within the gDNA of *K. pneumoniae* isolates, particularly the five isolates from Al-Karama hospital, in contrast to using the P13 primer. Although the bands electrophoresed on the agarose gel look comparable at first glance, there are variances in intensity along with the absence of some bands from some isolates. The dendrogram demonstrates a 75% genetic similarity between isolates 1 and 5 from Al-Karama hospital, as well as a similarity of more than 70% between isolates 7 and 8 from Al-Zahraa hospital (Figure 2). When the other DNA samples (isolates 9 to 16) from Al-Zahraa hospital were amplified by RAPD-PCR using OPX-4 primer, the dendrogram revealed the presence of two genetically related clusters, the first of which revealed 70% similarity between isolates 12 and 15. While the isolates no. 9 and no. 10 from Al-Zahraa hospital had 90% similarity (Figure 3).

Table 1. List of primers, their sequences, GC%, and melting temperature.

No.	Primer name	Tm	GC%	Sequence 5'-3'
1	P13	34.0	70.0	ACCGCCTGCT
2	OPX-04	34.0	70.0	CCGCTACCGA
3	OPY-01	32.0	60.0	GGTGGCATCT

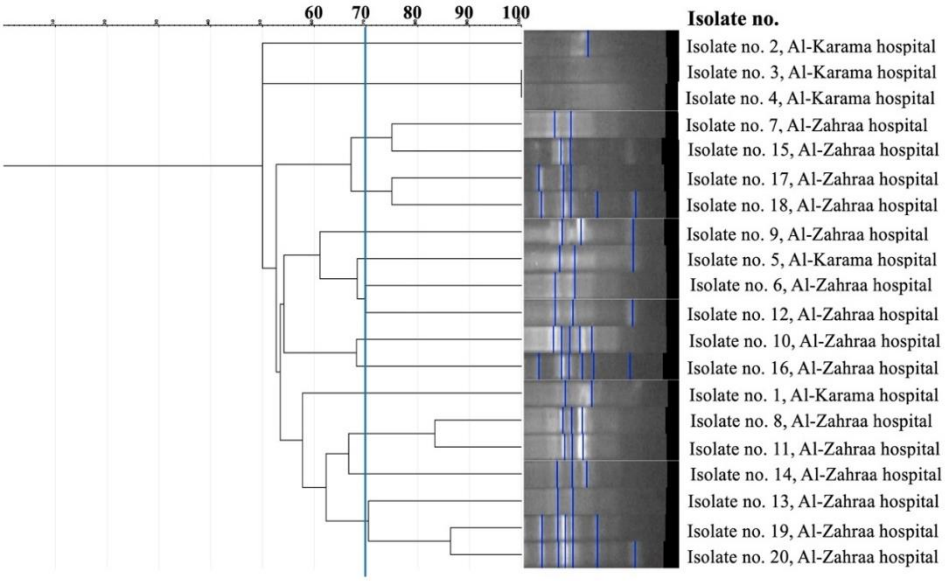

Figure 1. Dendrogram of the electrophoresed gDNA of *K. pneumoniae* isolates amplified by RAPD-PCR using P13 primer. The dendrogram was created by Dice coefficient and the UPGMA clustering method.

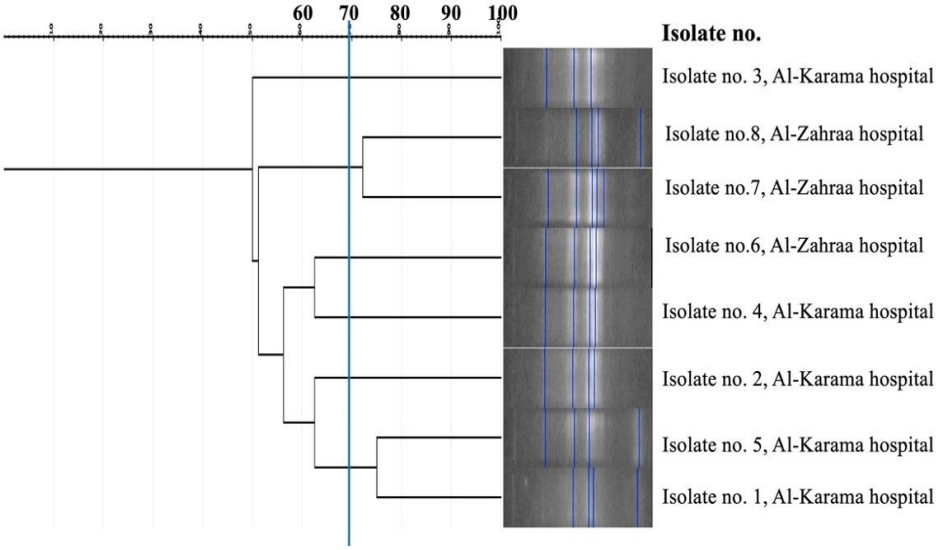

Figure 2. Dendrogram of the electrophoresed gDNA of *K. pneumoniae* isolates (no. 1-8) amplified by RAPD-PCR using OPX-04 primer. The dendrogram was created by Dice coefficient and the UPGMA clustering method.

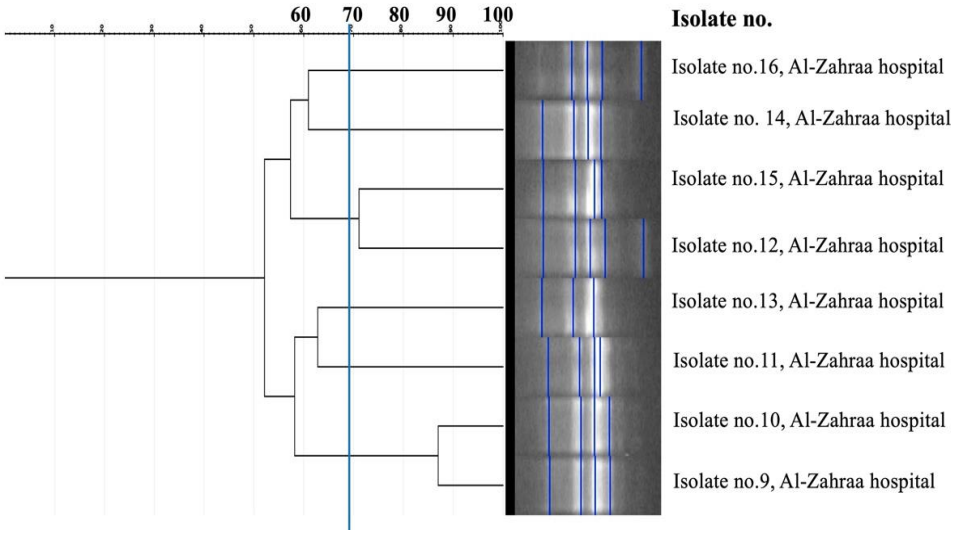

Figure 3. Dendrogram of the electrophoresed gDNA of *K. pneumoniae* isolates (no. 9-16) amplified by RAPD-PCR using OPX-04 primer. The dendrogram was created by Dice coefficient and the UPGMA clustering method.

RAPD-PCR based on the OPY-01 primer

When the isolated DNA of samples 1–8 was amplified with the OPY-01 primer and then analyzed on an agarose gel electrophoresis, just a few bands appeared in isolates 2–8. While isolate no. 1 had amplification of four bands, making it different from the other isolates from the same hospital (Figure 4). The dendrogram analysis of the eight isolates revealed a genetic link of 70% similarity between isolate 4 from Al-Karama hospital and isolate 8 from Al-Zahraa hospital (Figure 4).

Unlike the DNA isolated from samples 1–8, the DNA of isolates 9–16 and amplified with the same octamer primer (OPY-01) indicated amplification of multiple bands, i.e. four distinct bands, in all the analyzed DNA (Figure 5). However, disparities in band intensity were noticed across the isolates. Figure 5 is a dendrogram that shows around 85% genetic similarity between isolates 9 and 10 from Al-Zahraa hospital.

DISCUSSION

The incidence of multidrug-resistant (MDR) *Klebsiella pneumoniae* contamination in burn infections has increased in recent years (Aljanaby & Alhasnawi, 2017; Sadeq & Lafta, 2024). Genotyping is a valuable approach for investigating nosocomial infections (Lagha *et al.,* 2021). Among the methods used for the bacterial genotyping is the pulsed-field gel electrophoresis (PFGE) assay, which is more precise, epidemiological, and the gold standard technique for molecular typing of pathogens.

It has been applied to track the origin and monitor the dissemination patterns of various *K. pneumoniae* strains in the hospital environment (Poh *et al.,* 1993; Hansen *et al.,* 2002; Teimour pour *et al.,* 2020). However, PFGE has drawbacks, including the fact that it is labor-intensive and time-consuming, necessitating the use of specialized equipment, restriction endonucleases, and expert workers. In comparison, RAPD-PCR has been widely used as an epidemiological tool and has proven to be an effective method for distinguishing between different *K. pneumoniae* isolates (Tribuddharat *et al.,* 2008). It can identify polymorphisms throughout the genome (Gurakan *et al.,* 2008).

In this study, the 20 *K. pneumoniae* isolates; five from Al-Karama hospital and fifteen from Al-Zahraa hospital, revealed various banding patterns on the agarose gel electrophoresis performed following RAPD-PCR using octamer primers. The choice of suitable primers is a critical stage in the development of RAPD-PCR

technology (Lanzone *et al.,* 2016). The P13 primer was highly discriminating for the isolates under study. The OPX-04 primer was also able to distinguish between the isolates of the two hospitals, revealing both genetic closeness and genomic divergence. The OPY-01 primer produced numerous bands of varying sizes. Thus, the findings of these primers may be attributable to the isolates' genetic diversity. In this regard, the current data correspond with the observations of many authors who found *K. pneumoniae* be highly heterogeneous due to the occurrence of variations in nucleotide sequences (Al-Marzooq *et al.,* 2014; Ashayeri-Panah *et al.,* 2013; 2014; Wasfi *et al.,* 2016). These findings provide useful information on the molecular epidemiology of *K. pneumoniae* isolates circulating in two hospital settings. The genotypic polymorphism seen among isolates from the same hospital may indicate the isolates' genetic variety or their different origins (Akya *et al.,* 2018; Elahi *et al.,* 2019). Bacterial strains can spread in the hospital environment through a variety of means, including contaminated devices and equipment, cleaners, and even hospital workers (Elahi *et al.,* 2019). These bacterial strains can cause nosocomial infections, and there are limited effective medicines available to treat them (Elahi *et al.,* 2019).

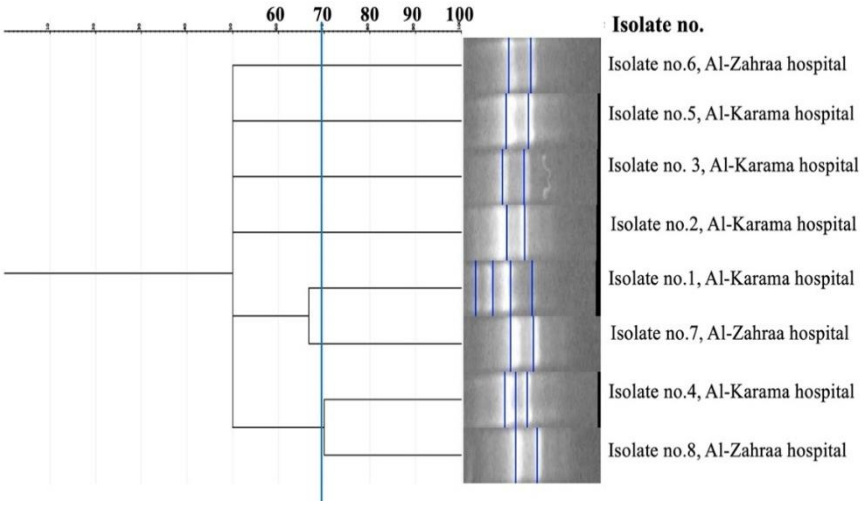

Figure 4: Dendrogram of the electrophoresed gDNA of *K. pneumoniae* isolates (no. 1-8) amplified by RAPD-PCR using OPY-01 primer. The dendrogram was created by Dice coefficient and the UPGMA clustering method.

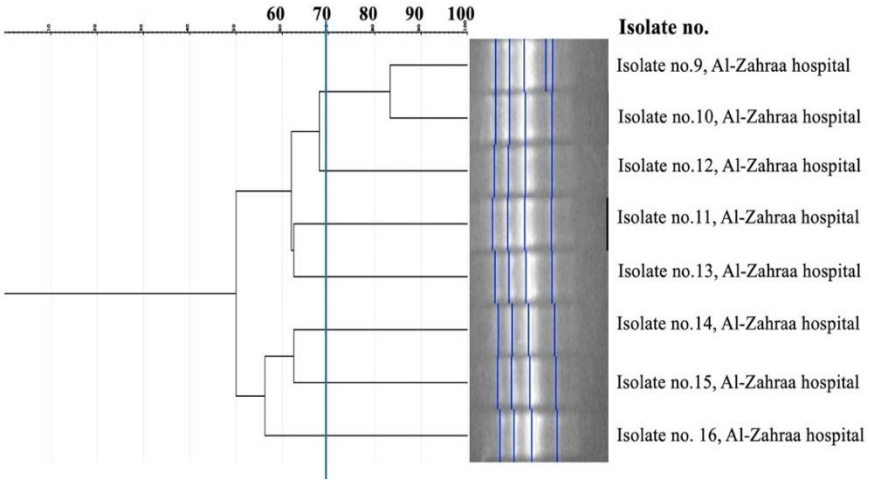

Figure 5. Dendrogram of the electrophoresed gDNA of *K. pneumoniae* isolates (no. 9-16) amplified by RAPD-PCR using OPY-01 primer. The dendrogram was created by Dice coefficient and the UPGMA clustering method.

Nevertheless, close genetic relatedness across numerous isolates, particularly those isolated from the same hospital, was also reported in the current investigation. It has been demonstrated that the occurrence of certain strains with identical genotypes in clusters might indicate a common source for these strains and may represent clonal transmission within hospital environments, particularly in intensive care units and burn wards (Elahi *et al.,* 2019). Other studies have reported lower diversity and higher genotype similarity among isolates (Christian *et al.,* 2010; Dedeic-Ljubovic *et al.,* 2010; Mshana *et al.,* 2023; Tijet *et al.,* 2014). One explanation could be the fact that the majority of isolates in the aforementioned investigations came from limited sources in one hospital (Akya *et al.,* 2018; Elahi *et al.,* 2019).

In this study, the OPY-01 primer was able to detect some similarities across isolates from various hospitals. It has been suggested that strains from different hospitals with similar genotypic patterns can be disseminated via patients commuting between hospitals, particularly in intensive care units and burn wards (Akya *et al.,* 2018). As a result, Al-Kut hospitals must build more efficient infection control methods to minimize the spread of highly resistant bacterial strains inside the medical setting. However, one of the study's shortcomings is the difficulty of employing the PFGE test, which has long been used to genotype *K. pneumoniae* and other bacteria. Thus, this study recommends the use of multiplex primers (the three primers combined) in multiplex PCR after optimization to boost discriminating power and improve the study's outcome.

CONCLUSION

According to the findings of this investigation, burn wound isolates of *K. pneumoniae* acquired from patients hospitalized to two hospitals exhibited a significant genetic diversity. These isolates might circulate concurrently throughout the hospital setting. As a result, better clinician awareness and improved laboratory testing are critical to reducing treatment failure and preventing the spread of nosocomial bacteria with antibiotic resistance.

ACKNOWLEDGEMENTS

We would like to thank and acknowledge Dr. Zahraa E. Sadeq for providing the clinical isolates used in this study.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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