High prevalence of *Klebsiella pneumoniae* carbapenemase-mediated resistance in *K. pneumoniae* isolates from Egypt

L. Metwally,[1] N. Gomaa,[1] M. Attallah[2] and N. Kamel[3]

معدل انتشار مرتفع للمقاومة للكلبسيلا الرئوية بتواسط إنزيم كاربابينيماز في مُستفردات الكلبسيلا الرئوية في مصر

لبنى متولي، ناهد جمعة، مكرم عطا الله، نها كامل

الخلاصـة: إن البزوغ والانتشار السريع لمستفردات الكلبسيلا الرئوية المقاومة للمضادات الحيوية والتي تحتوي على الجين bla_{kpc} الذي يرمز لإنتاج إنزيم كاربابينيماز قد أدى إلى تعقيد التدبير العلاجي للمرضى المصابين بالعدوى. وقد أجرت الباحثات هذه الدراسة في مستشفى للرعاية الثالثية في مصر باستخدام مقايسة التفاعل السلسلي للبوليميراز في الزمن الحقيقي لاختبار مستفردات الكلبسيلا الرئوية التي لا تستجيب للإرتابنيم لكشف وجود الجين bla_{kpc} ولمقارنة النتائج مع اختبار هدج المعدَّل. وقد درست الباحثات الاستجابة للمضادات الحيوية بالطرق المعيارية، وقُمْنَ بتفسيرها باتباع نقاط الفصل القديم المعتمدة لدى معهد المعايير السريرية والمختبرية (M100-S19) بالنسبة لمركبات كاربابينيم، إلى جانب نقاط الفصل المنقَّحة (M100- S22). وشملت الدراسة 45 مستفردة غير مزدوجة للكلبسيلا الرئوية أخذت من عينات سريرية مختلفة، واتضح للباحثات أن معدل انتشار مرتفعاً للمستفردات غير المستجيبة للإرتابينيم (44.4٪) قد تم تسجيله باستخدام نقاط الفصل المنخفضة والجديدة المعتمدة لدى معهد المعايير السريرية والمختبرية، كما تأكد للباحثات وجود الجين bla_{kpc} في 14 من بين 20 مستفردة (70٪)، ويعزى ارتفاع معدل انتشار عدم الاستجابة للإرتابينيم في مستشفى الرعاية الثالثية في مصر على الأغلب إلى آليات المقاومة التي تتواسطها إنزيمات كاربابينيماز في مستفردات الكلبسيلات الرئوية.

ABSTRACT The emergence and rapid spread of antibiotic-resistant *Klebsiella pneumoniae* isolates harbouring the bla_{KPC} gene that encodes for carbapenemase production have complicated the management of patient infections. This study in a tertiary care hospital in Egypt used real-time PCR assay to test ertapenem-nonsusceptible isolates of *K. pneumoniae* for the presence of the bla_{KPC} gene and compared the results with modified Hodge test. Antibiotic sensitivity was performed by standard methods, and interpreted following both the old CLSI breakpoints (M100-S19) for carbapenems and the revised breakpoints (M100-S22). From the 45 non-duplicate isolates of *K. pneumoniae* recovered from different clinical specimens, a high prevalence of ertapenem-nonsusceptible isolates (44.4%) was reported using the new lower CLSI breakpoints. The bla_{KPC} gene was confirmed in 14/20 (70.0%) of these isolates. The high prevalence of ertapenem nonsusceptibility at a tertiary care hospital in Egypt was predominantly attributed to *K. pneumoniae* carbapenemase-mediated resistance mechanisms in *K. pneumoniae* isolates.

Prévalence élevée de la résistance de *Klebsiella pneumoniae* médiée par les carbapénèmases dans des isolats de *K. pneumoniae* en Égypte

RÉSUMÉ L'émergence et la propagation rapide des souches de *Klebsiella pneumoniae* résistantes aux antibiotiques et porteuses du gène bla_{KPC} codant la production de carbapénèmases ont compliqué la prise en charge des infections des patients. La présente étude menée dans un hôpital de soins tertiaires en Égypte a utilisé la méthode de PCR en temps réel pour évaluer la présence du gène bla_{KPC} dans les isolats de *K. pneumoniae* non sensibles à l'ertapénème, puis a comparé les résultats à l'aide du test de Hodge modifié. La sensibilité aux antibiotiques a été évaluée à l'aide des méthodes standards, puis a été interprétée selon les anciens seuils du *Clinical and Laboratory Standards Institute* (M100-S19) pour les carbapénèmes et selon les seuils révisés (M100-S22). Après l'analyse des 45 isolats non-dupliqués de *K. pneumoniae* prélevés à partir de différents échantillons cliniques, une prévalence élevée d'isolats non sensibles à l'ertapénème (44,4 %) a été rapportée selon les nouveaux seuils plus bas du *Clinical and Laboratory Standards Institute*. La présence du gène bla_{KPC} a été confirmée dans 14 isolats sur 20 (70,0 %). La forte prévalence de la non sensibilité à l'ertapénème dans un hôpital de soins tertiaires en Égypte était principalement imputable aux mécanismes de résistance médiés par les carbapénèmases dans les isolats de *K. pneumoniae*.

[1]Department of Microbiology; [3]Department of Clinical Pathology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt (Correspondence to L. Metwally: lobna.metwally@gmail.com). [2]Department of Microbiology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

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Introduction

Klebsiella pneumoniae carbapenemases (KPCs) are Ambler class A plasmid-encoded enzymes that are capable of hydrolyzing all beta-lactam antibiotics, including monobactams, extended-spectrum cephalosporins and carbapenems [1,2]. Originally described in 2001 [3], pathogens harbouring these antibiotic-resistance enzymes have been reported from the United States of America (USA) [4–7], France, China, Sweden, Norway, Colombia, Brazil, Scotland, Germany and Spain [8–11]. Epidemic situations have also been reported in Israel and Greece [12,13]. An important challenge to developing a standardized definition of bacterial isolates resistant to carbapenems is a recent (mid-2010) change in the Clinical and Laboratory Standards Institute (CLSI) interpretative criteria (breakpoints) for determining susceptibility to carbapenems among Enterobacteriaceae [14,15]. These new recommendations lowered the breakpoints and removed the requirement for testing for carbapenemases, e.g. by modified Hodge test (MHT), to determine susceptibility. However, based on clinical and microbiological data, ertapenem breakpoints were modified again in January 2012 (M100-S22) by doubling the dilution (to ≤ 0.5 µg/mL) [16].

In addition to beta-lactam/carbapenem resistance, nonsusceptible organisms can carry genes that confer high levels of resistance to many other antimicrobials, often leaving very limited therapeutic options [17,18]. The bla_{KPC} gene encodes for KPC enzyme production. Although carbapenemases have been identified in many species of Enterobacteriaceae, *K. pneumoniae* remains the most common organism carrying resistance-encoding genes [2]. Carbapenem resistance in *K. pneumoniae* may also be due to production of other carbapenemases [19] or to changes in outer membrane porin proteins [20], often combined with production of an extended-spectrum beta-lactamase, AmpC or both [19,21].

Molecular detection of the bla_{KPC} gene by polymerase chain reaction (PCR) assay provides laboratories with a means to quickly identify the presence of this important resistance determinant [22,23]. Considering the demonstrated potential for rapid horizontal and vertical transmission of the bla_{KPC} gene, prompt recognition is important to controlling the spread of KPCs. In the present study we describe a real-time PCR assay to detect all variants of the bla_{KPC} gene and the use of this assay to test clinical isolates of *K. pneumoniae*. We also tested ertapenem-nonsusceptible isolates using MHT.

Methods

Study isolates

A prospective study was conducted over a period of 6 months (June 2011 to December 2011) at the Suez Canal University hospital, Ismailia, Egypt. A total of 45, single-patient *K. pneumoniae* isolates were included in the study. These isolates were recovered from urine (n = 13), blood (n = 8), respiratory tract (n = 12) and other clinical sites (n = 12) from patients admitted to the intensive care unit and different wards of the hospital. Full identification was carried out using the API 20E system (bioMérieux).

Ethical approval to perform the study was obtained from the ethics committee in the Faculty of Medicine, Suez Canal University and the management board of the hospital. All the included patients consented to the collection of specimens before the study was initiated.

Susceptibility testing

Antibiotic susceptibility testing was determined using the modified Kirby–Bauer method following the CLSI guidelines. The following antimicrobial agents were included in the panel: ampicillin, amoxicillin/clavulanic acid, ceftriaxone, cefepime, cefazolin, cefoxitin, ciprofloxacin, gentamicin, tobramycin, imipenem, ertapenem, meropenem, trimethoprim/sulfamethoxazole, piperacillin, piperacillin/tazobactam and tobramycin (Oxoid).

Isolates were further subjected to minimum inhibitory concentration (MIC) testing for imipenem and meropenem using the Oxoid MIC evaluator strip (Thermo Fisher Scientific) and for ertapenem using the gradient strip E-test (bioMérieux); boxes were allowed to equilibrate at room temperature for at least 1 h before opening. For all isolates the inocula for strip tests were matched to a 0.5 McFarland standard. Results were read in accordance with the manufacturers' directions and interpreted following both the old CLSI M100-S19 breakpoints and the revised breakpoints in the M100-S22 document issued in January 2012 [14–16].

Suspension of a known KPC-producing isolate [*K. pneumoniae* American Type Culture Collection (ATCC) BAA-1705], recovered on the MacConkey agar, was used as quality control strain. A second carbapenem-susceptible *K. pneumoniae* (ATCC 700603) was used as negative control.

Stocks of 20 distinct single-patient *K. pneumoniae* isolates representing different antibiogram patterns and showing MIC ≥ 1 µg/mL (n = 20) for ertapenem, using the revised carbapenem breakpoints (M100–S22, January 2010), were tested for carbapenemases by MHT and stored in tryptic soy broth with 20% glycerol at –20 °C until further testing by bla_{KPC} real-time PCR.

Detection of bla_{KPC} by real-time PCR

Fresh, well-isolated test colonies grown on sheep-blood agar plates following overnight incubation were used for DNA extraction using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol. Briefly, a 2.0 McFarland standard bacterial

suspension was prepared in saline, and bacterial DNA was extracted from 200 µL (1.2 × 10^8 colony forming units) of the suspension. Extracted bacterial DNA was eluted from the columns in 100 µL elution buffer and stored at –20 °C.

The TaqMan real-time KPC PCR assay uses previously published primers and probes which detect all currently described KPC variants [24]. The sequences were as follows: for the KPC forward primer, 5′-GCG GAA CCA TTC GCT AAA CTC GAA-3′; for the KPC reverse primer, 5′-AGA AAG CCC TTG AAT GAG CTG CAC-3′; and for the KPC probe, 5′-/6-FAM/ ATA CCG GCT CAG GCG CAA CTG TAA GTT A/6-TAMRA/-3′ (where 6-FAM represents 6-carboxy-fluorescein and 6-TAMRA represents 6-carboxytetramethylrhodamine).

Real-time PCR was performed with 2 µL template DNA in a total reaction volume of 10 µL containing 1× LightCycler FastStart DNA master hybridization probe reagent (Roche Diagnostics), 3.5 mM $MgCl_2$, and 2 µM of primers for bla_{KPC} and the TaqMan probe.

A negative control consisting of the reaction mixture and water (in place of template DNA) was added in each run. In addition to negative controls, a reference *K. pneumoniae* strain (ATCC BAA-1705) was selected as the positive control.

The LightCycler 2.0 instrument (Roche Diagnostics) was used for the amplification and detection of the bla_{KPC} gene using the following PCR cycling conditions; after an initial denaturation step of 3 min at 95 °C, a 2-step PCR procedure was used consisting of 30 s at 95 °C and 1 min at 60 °C for 45 cycles.

Data were obtained during the annealing period. Fluorescence was measured once every cycle immediately after the 60 °C incubation (extension step). Fluorescence curves were analysed with the *LightCycler* software, version 4.0. The results were expressed by determination of the threshold cycle (Ct) value which marked the cycle at which the fluorescence of the sample became significantly different from the baseline signal. A sample was regarded as positive when the *LightCycler* software determined a Ct in the quantification analysis screen.

When analysing the results, it is important to only consider amplification between 10–35 cycles as positive. Amplification prior to 10 cycles means the template should be diluted before repeating. Amplification after 35 cycles can indicate trace contamination. The no template (water) control should not yield a product (Ct > 40). PCR positive isolates with reduced ertapenem MIC were considered to be KPC positive.

Detection of KPC by the MHT

Isolates that were nonsusceptible to ertapenem (i.e. resistant and intermediate isolates) were also tested by the MHT previously described [25]. Briefly, a 0.5 McFarland suspension of *Escherichia coli* (ATCC 25922), was used to prepare a lawn culture on a Mueller–Hinton agar plate (Becton Dickinson), and a 10 µg ertapenem susceptibility disk (Oxoid) was placed in the centre of the test area. Test isolates were subcultured onto sheep-blood agar plates (Becton Dickinson) to establish pure cultures. The isolate was then streaked in a straight line from the edge of the disk to the edge of the plate and was incubated overnight at 35 °C in ambient air. After 24 hours of incubation, the plate was examined for a cloverleaf-shaped indentation at the intersection of the test organism and the *E. coli* ATCC 25922 within the zone of inhibition. The presence of a cloverleaf-shaped indentation was considered MHT positive.

Results

By using current breakpoints (M100-S22) for carbapenem interpretation, 20 out of 45 *K. pneumoniae* isolates (44.4%) were reported as nonsusceptible (intermediate and resistant) to ertapenem (Table 1). However, when the old 2009 breakpoints were used, ertapenem interpretation classified only 15 (33.3%) of isolates as nonsusceptible and 30 (66.7%) as susceptible. Of the 5 isolates that was counted as susceptible by the 2009 guidelines yet nonsusceptible by the new guidelines, 3 isolates were positive for the bla_{KPC} gene; these isolates were susceptible to meropenem and imipenem. Among the isolates tested, 40.0% and 37.8% were nonsusceptible to imipenem and meropenem respectively at the new CLSI resistance breakpoint of ≥ 2 µg/mL for both drugs (Table 1).

Table 1 Minimum inhibitory concentration results for carbapenem antibiotics on *Klebsiella pneumoniae* isolates (*n* = 45) using different Clinical and Laboratory Standards Institute (CLSI) breakpoints

Antibiotic agent	Older breakpoints[a]				Current breakpoints[b]			
	Susceptible		Nonsusceptible[c]		Susceptible		Nonsusceptible[c]	
	No.	%	No.	%	No.	%	No.	%
Imipenem	34	75.6	11	24.4	27	60.0	18	40.0
Meropenem	33	73.3	12	26.7	28	62.2	17	37.8
Ertapenem	30	66.7	15	33.3	25	55.6	20	44.4

[a]*CLSI M100-S19 criteria [14,15];* [b]*CLSI M100-S22 criteria [16];* [c]*Intermediate and resistant.*

Table 2 **Results of modified Hodge test (MHT) and polymerase chain reaction (PCR) assay for bla_{KPC} gene on nonsusceptible *Klebsiella pneumoniae* isolates (*n* = 20)**

Modified Hodge test results	Polymerase chain reaction results		Total
	PCR+ve	PCR–ve	
MHT+ve	14	3	17
MHT–ve	0	3	3
Total	14	6	20

+ve = positive; –ve = negative.

Real-time KPC PCR assay results were used to confirm that carbapenem resistance in *K. pneumoniae* isolates was due to production of a KPC. Of the 20 *K. pneumoniae* isolates with reduced susceptibility to ertapenem (defined as ≥ 1 µg/mL), according to the revised clinical breakpoints, 14 isolates were found positive for KPCs by MHT and by PCR detection of the bla_{KPC} gene (Table 2). Of the remaining 6 isolates that were negative by PCR, 3 isolates were positive by MHT.

Discussion

The emergence and rapid spread of antibiotic-resistant *K. pneumoniae* isolates harbouring the bla_{KPC} gene that encodes for carbapenemase production have complicated the management of patients' infections [1,2]. To our knowledge, this is the first published report of KPC-producing *K. pneumoniae* isolated from patients at a tertiary care hospital in Egypt. In our study we used ertapenem to screen for carbapenemases, as ertapenem is the least active carbapenem against KPCs [26] and as the use of this drug in automated or manual susceptibility testing has been found to be a highly sensitive method for the detection of KPCs [26,27]. Despite the limited number of isolates included, we were able to show a high prevalence of ertapenem non-susceptibility, accounting for 44.4% of *K. pneumoniae* isolates tested. This high prevalence reflected the new lower CLSI breakpoints for carbapenems. When the previous CLSI breakpoints for ertapenem were used (resistant > 4 µg/mL; susceptible ≤ 2 µg/mL), only 33.3% would be counted as nonsusceptible. A high prevalence of ertapenem resistance was similarly reported by many investigators in different countries [5,19]. For instance, in a study from China none of the 77 clinical isolates collected from 2002 to 2009 were susceptible to ertapenem and only 6.5% and 1.3% of isolates were susceptible to imipenem and meropenem respectively [28].

Of the 5 isolates that were counted as ertapenem-susceptible by the old CLSI M100-S19 breakpoints but nonsusceptible by the revised breakpoints, 3 isolates were positive for bla_{KPC} genes and MHT, signifying the improved rate of detection of KPC-meditated resistance when using the new CLSI breakpoints. Likewise, the new breakpoints increased the proportion of isolates counted as nonsusceptible to imipenem and meropenem (to 40.0% and 37.8% respectively), although these were less than for ertapenem.

We also described in this study, a real-time PCR designed to detect and characterize genes encoding all KPC variants. Using this assay, we documented for the first time in Egypt the presence of isolates producing KPCs. Isolates with ertapenem MIC ≥ 1 µg/mL were further investigated to determine the prevalence of KPC enzymes. We were able to confirm the presence of bla_{KPC} genes in 14 (70.0%) of ertapenem-nonsusceptible isolates, which comprised 31.1% of all isolates tested, indicating that the increased prevalence of ertapenem non-susceptibility was predominantly attributed to KPC-mediated resistance mechanisms in *K. pneumoniae*. Prevalence rates of KPC-positive *K. pneumoniae* isolates of > 30% have been recorded in some institutions in the eastern USA, in association with nosocomial outbreaks [27].

Our results suggest performing confirmatory testing for the presence of KPC for all ertapenem-resistant bacteria. All KPC-producing bacteria were also MHT positive, indicating the usefulness of doing this phenotypic testing. However, due to the more rapid turnaround time of PCR assays, this assay might be more suitable as an initial screening test for detecting KPC-mediated carbapenem resistance. On the other hand, PCR is more technically challenging, prone to inhibition and may miss new variants of KPC arising from genetic mutation

Of the ertapenem-nonsusceptible isolates 6 were negative by real-time bla_{KPC} PCR and, of those, 3 isolates were positive by MHT. Two possibilities exist that may explain these 3 MHT-positive/KPC-PCR-negative isolates. First, as reported by Schechner et al. KPC PCR could be falsely negative due to inhibitory substances in the reaction or to technical inexperience of the laboratory [29]. However, the most probable reason could be the presence of other carbapenemases, such as the metallo-beta-lactamases and the member of the *Serratia marcescens* (SME) family of carbapenem-hydrolyzing beta-lactamases, SME-1, which can produce a positive result for MHT but negative for bla_{KPC}. So although the new CLSI recommendations lowered the breakpoints of carbapenems and removed the requirement for testing for carbapenemase (e.g. MHT) to determine susceptibility [15], performing MHT as an adjunct to KPC PCR may increase the likelihood of detecting other carbapenemases. Furthermore, the current recommendation is to still

to perform MHT for infection control and epidemiological purposes.

Our study had some limitations. First, the number of isolates included in the study was limited by the low incidence of *K. pneumoniae*-associated infections in our institution during the study; nonetheless, the available results provided robust pilot data. Secondly, molecular detection of bla_{KPC} genes was further limited to isolates nonsusceptible to ertapenem. However, this did not substantially compromise our study findings, especially when using the new lower CLSI breakpoints for interpretation. Thirdly, we did not screen our isolates for other resistance determinants, such as AmpC or outer membrane proteins, owing to limited funding available.

In summary, our data showing an increased prevalence of ertapenem-nonsusceptible *K. pneumoniae* isolates partly reflects lowering of clinical breakpoints but also indicates the spread of carbapenemases, principally KPC types, in Suez Canal University hospital, Egypt. Confirmatory testing for the presence of KPCs is required for all ertapenem-resistant bacteria. Real-time PCR assay described here provides a useful tool to rapidly and accurately detect bla_{KPC}-positive bacteria, which is an important step in controlling their spread.

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