Infection and Drug Resistance

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ORIGINAL RESEARCH

The First Egyptian Report Showing the Co-Existence of bla_{NDM-25}, bla_{OXA-23}, $bla_{OXA-181}$, and bla_{GES-1} Among Carbapenem-Resistant *K. pneumoniae* Clinical Isolates Genotyped by BOX-PCR

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Background and Objective: The emergence of carbapenem-resistant *K. pneumoniae* (CRKP) continues to escalate and is alarming because of the emergence of pan drug-resistant strains. The objective of this study was to investigate the existence of 12 carbapenemase genes among CRKP clinical isolates.

Methods: Ninety-six *Klebsiella* spp. clinical isolates were collected. The isolates were identified phenotypically and genotypically. These isolates were screened for susceptibility to 24 different antibiotics. The modified Hodge test (MHT) and the Carba Nordmann/Poirel (NP) test were used to phenotypically screen carbapenem-resistant strains for carbapenemase production. Phenotypic characterization of carbapenemases was performed using the combined disk synergy test (CDST). Additionally, the presence of 12 carbapenemase genes in CRKP isolates was investigated. The DNA sequence of bla_{NDM} and bla_{GES} genes was determined. The BOX-PCR technique was used to determine the clonal relationship between CRKP isolates.

Results: All carbapenem-resistant isolates were related to *K. pneumoniae*. Susceptibility testing showed that 19.79% (19/96) of the collected isolates were carbapenem-resistant. Of the CRKP isolates, 68.42% (13/19) tested positive for the MHT and Carba NP test. CDST showed that 42.11% (8/19), 63.16% (12/19), 47.37% (9/19), and 73.68% (14/19) of the CRKP isolates tested positive for the inhibitory effect of clavulanic acid, sulbactam, phenylboronic acid, and tazobactam, respectively, while 84.21% (16/19) and 68.42% (13/16) tested positive for the inhibitory effect of EDTA and mercaptopropionic acid, respectively. It was found that 10.53% (2/19) of the isolates tested positive for the inhibitory effect of sodium chloride. Molecular investigation of carbapenemases showed that 26.32% (5/19), 73.68% (14/19), 21.05% (4/19), 10.53% (2/19), and 5.26% (1/19) of the isolates tested positive for bla_{NDM}, bla_{OXA-48}, $bla_{OXA-181}$, bla_{OXA-51}, and bla_{OXA-23}, respectively. None of the isolates tested positive for bla_{OXA-40} and bla_{OXA-58}. Two allelic variants of bla_{NDM} (bla_{NDM-1} and bla_{NDM-25}) were detected. BOX-PCR revealed high clonal relatedness between CRKP isolates.

Conclusion: MHT was more sensitive than Carba NP test for evaluating carbapenemase production and class D carbapenemase genes were the most prevalent of the 12 carbapenemase genes that were evaluated.

Keywords: carbapenemases, CRKP, BOX-PCR, bla_{NDM-25}, MHT, CDST

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Introduction

Carbapenems are one of the most important groups of antibiotics used to treat bacterial infections caused by multiple drug-resistant (MDR) Gram-negative enteric isolates.[1]

Irrational and widespread use of carbapenems in intensive care units (ICUs) and other hospital settings has led to the emergence and spread of carbapenem-resistant strains that can be treated only by antimicrobial agents such as tigecycline and colistin[1,2] that are considered to be last resort for treating carbapenem-resistant *Enterobacteriaceae* (CRE).[3]

Resistance to carbapenems in Gram-negative bacteria is mediated by three mechanisms, including (i) production of hydrolytic enzymes known as carbapenemases, (ii) extrusion of carbapenems from the interior of bacterial cells by efflux pumps, and (iii) downregulation of the outer membrane proteins (OMPs) that are involved in transmembrane passage of carbapenems to reach their targets inside bacterial cells.[1,4]

According to the definition proposed by the Center for Disease Control and Prevention (CDC), *K. pneumoniae* is considered to be MDR when it exhibits resistance to at least one agent in three or more classes of antimicrobial agents.[5] Extensive drug resistance (XDR) corresponds to MDR isolates that develop resistance to all antibiotic groups except for two or fewer classes.[6] Pan drug resistance (PDR) corresponds to XDR isolates that develop resistance to all antibiotics, including polymyxin and tigecycline.[4,6]

Production of carbapenemases represents the most important carbapenem resistance mechanism among Gram-negative *Enterobacteriaceae* in which the genes encoding for these enzymes are easily transferred between bacterial flora in hospitals, which can lead to serious outbreaks within different hospital settings.[7]

According to their active sites, carbapenemases are classified into two main classes: serine carbapenemases and metallo-carbapenemases.[8,9] Serine carbapenemases are further classified into two molecular groups according to the Ambler classification of β-lactamases: class A serine carbapenemases (CASCs) and class D serine carbapenemases, which are also known as OXA-type carbapenemases (OTCs) in which these carbapenemases are actually oxacillinases.[10]

In clinical settings, CASCs can be inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. In contrast, OTCs are poorly inhibited by the aforementioned β-lactamase inhibitors.[11]

Class B carbapenemases are metallo-β-lactamases in which the active site requires zinc ions as a cofactor. However, metallo-carbapenemases cannot be clinically inhibited but can be inhibited in vitro by chelating agents such as ethylene diamine tetraacetic acid (EDTA) and mercaptopropionic acid (MPA).[12,13]

BOX-PCR is a DNA fingerprinting technique that detects repetitive and highly conserved DNA elements located on the bacterial chromosome.[14] These repeated conserved DNA elements were termed BOX dispersed-repeat motifs that were first identified on the chromosome of *Streptococcus pneumoniae*. It is now known that the BOX-motif is commonly found in many bacterial species.[14,15]

BOX-PCR detects a specific form of repetitive extragenic palindromic (rep) sequences that consist of three different regions called boxA, boxB, and boxC that consist of 59, 45, and 50 base pairs, respectively. BOX-PCR is used to discriminate between clinical isolates of the same genus in order to track genetic differences between them.[14]

To the best of our knowledge, there are no published studies from Egypt regarding investigation of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, and $bla_{\text{OXA-181}}$ in CRKP clinical isolates, and thus the current study was performed to investigate the existence of these carbapenemase encoding genes in CRKP clinical isolates recovered from Egyptian patients and to identify the clonal relatedness between these isolates.

Patients and Methods

Bacterial Strains

Nineteen CRKP isolates were selected from a total of 96 *Klebsiella* spp. clinical isolates that had been recovered as a part of routine microbiological laboratory procedures at the hospital involved in the present study. The CRKP clinical isolates were collected from 17 cases (9 males and 8 females) who had been admitted to different hospital departments. The 19 CRKP clinical isolates were recovered from sputum (n = 3), urinary catheter (n = 1), urine (n = 6), wound (n = 7), and central line catheter (n = 2).

Isolation and Identification of *K. pneumoniae* Isolates

All recovered *Klebsiella* spp. clinical isolates were primarily isolated on MacConkey's agar (Oxoid, UK) then on eosin methylene blue (EMB) agar (Scharlau, Spain). The isolated strains were identified phenotypically using API 20E (Biomerieux, France) and were confirmed genotypically based on the amplification of the *K. pneumoniae phoE* gene as previously described[16] using the primers and cycling conditions listed in Table 1.

Antimicrobial Susceptibility Testing (AST)

All *K. pneumoniae* clinical isolates were tested for susceptibility to 13 β-lactams, three different β-lactam/β-lactamase inhibitor combinations, and seven other agents representing

Table 1 Primers and Cyclic Conditions Used in PCR Targeting Three Classes of Carbapenemases and BoxA Region Among CRKP Clinical Isolates

Primer	Target Gene	Primer Sequence	Amplicon Size (bp)	Cycling Condition	Reference
KL_Iden	*phoE*	**F:** TGGCCCGCGCCCAGGGTTCGAAA **R:** GATGTCGTCATCGTTGATGCCGAG	368	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **60°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	16
BOX-AIR	BoxA region	CTACGGCAAGGCGACGCTGACG	-	Initial denaturation at 95°C for 15 min, then 35 cycles of 95°C for 1 min, **40°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	54
KPC	bla_{KPC}	**F:** GTATCGCCGTCTAGTTCTGC **R:** GGTCGTGTTTCCCTTTAGCC	637	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **55°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
GES	bla_{GES}	**F:** GCTTCATTCACGCACTATT **R:** CGATGCTAGAAACCGCTC	323	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **59°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
SME	bla_{SME}	**F:** ACTTTGATGGGAGGATTGGC **R:** ACGAATTCGAGCATCACCAG	551	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **66.7°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	56
IPM - Family	bla_{IPM}	**F:** GGAATAGRRTGGCTTAAYT **R:** GGTTTAAYAAARCAMCCACC	233	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **63°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
VIM-Family	bla_{VIM}	**F:** GTTTGGTCGCATATCGCAAC **R:** GAGCAAKTCYAGACCGCCC	591	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **55°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
NDM	bla_{NDM}	**F:** GGTTTGGCGATCTGGTTTTC **R:** CGGAATGGCTCATCACGATC	621	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **55°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
OXA-23	bla_{OXA-23}	**F:** GATCGGATTGGAGAACCAGA **R:** ATTTCTGACCGCATTTCCAT	501	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **66.6°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C	55
OXA-40	bla_{OXA-40}	**F:** GGTTAGTTGGCCCCCTTAAA **R:** AGTTGAGCGAAAAGGGGATT	249	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **66.6°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C	55
OXA-48	bla_{OXA-48}	**F:** GCTTGATCGCCCTCGATT **R:** GATTTGCTCCGTGGCCGAAA	281	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **60.5°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
OXA-51	bla_{OXA-51}	**F:** TAATGCTTTGATCGGCCTTG **R:** TGGATTGCACTTCATCTTGG	353	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **60.5°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
OXA-58	bla_{OXA-58}	**F:** AAGTATTGGGGCTTGTGCTG **R:** CCCCTCTGCGCTCTACATAC	599	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **60.5°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55
OXA-181	$bla_{OXA-181}$	**F:** ATGCGTGTATTAGCCTTATCG **R:** AACTACAAGCGCATCGAGCA	888	Initial denaturation at 95°C for 15 min, then 30 cycles of 95°C for 1 min, **55°C for 1min** and 72°C for 5 min and one cycle of final elongation at 72°C.	55

Notes: R=(A/G), Y=(C/T), K=(G/T) and M=(A/C). Boldface in this table indicates to forward and reverse primers and annealing temperature.
Abbreviations: A, adenine; G, guanine; C, cytosine; T, thymine; CRKP, carbapenem-resistant *K. pneumoniae*.

three different antibiotic classes. Susceptibility testing was performed using the modified Kirby–Bauer disc diffusion method[17] and the broth microdilution method.[18] Susceptibility results were interpreted based on guidelines from the Clinical Laboratory Standards Institute (CLSI).[19] *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as standard control strains.

Phenotypic Detection of Carbapenemase Production

Modified Hodge Test (MHT)

All selected CRKP clinical isolates were subjected to the MHT to detect carbapenemase production. MHT was performed as previously described[20] using a meropenem disk (10 µg) as a substrate. Test results were considered to be positive when the tested organism gave the characteristic clover leaf-like indentation around the meropenem disk as shown in Figure 1. A culture collection of *Acinetobacter baumannii* laboratory strains supplied by the microbiology laboratory at Taif University was used as a positive control.

Carba Nordmann/Poirel (CNP) Test

All CRKP isolates were investigated for carbapenemase production using the Carba NP test as previously described[21,22] in which both CNP test solutions (A) and (B) were prepared. Test results were considered to be positive when CNP solution (B) was converted from red to yellow color, while CNP solution (A) remained red as shown in Figure 1. A culture collection of *Acinetobacter baumannii* laboratory strains provided by the microbiology laboratory at Taif University was used as a positive control. *E. coli* ATCC 22925 was used as a negative control.

Phenotypic Characterization of Carbapenemases

Combined Disk Synergy Test (CDST)

Class A, B, and D carbapenemases expressed by the selected CRKP isolates were subjected to phenotypic characterization using CDST as previously described.[23] Phenyl boronic acid (PBA),[24] sulbactam, clavulanic acid (CA), and tazobactam were used as class A carbapenemase inhibitors.[25] EDTA[26] and MPA[27] were used as class B carbapenemase inhibitors. Sodium chloride was used as a class D carbapenemase inhibitor.[11,26]

Genotypic Detection of Carbapenemases

All CRKP clinical isolates were screened for 12 different genes encoding for class A, B, and D carbapenemases. Three class A carbapenemase genes (bla_{GES}, bla_{SME}, and bla_{KPC}), three class B carbapenemase genes (bla_{IPM}, bla_{VIM}, and bla_{NDM}), and six Class D genes (bla_{OXA-23}, bla_{OXA-40}, bla_{OXA-48}, bla_{OXA-51}, bla_{OXA-58}, $bla_{OXA-181}$) were screened using the primers and cycling conditions listed in Table 1.

Extraction of Total Genomic DNA

To investigate carbapenemase encoding genes, total genomic DNA (chromosomal and plasmid DNA) was extracted by a boiling method. Total genomic DNA was extracted as previously described[28] in which 3–5 colonies were picked

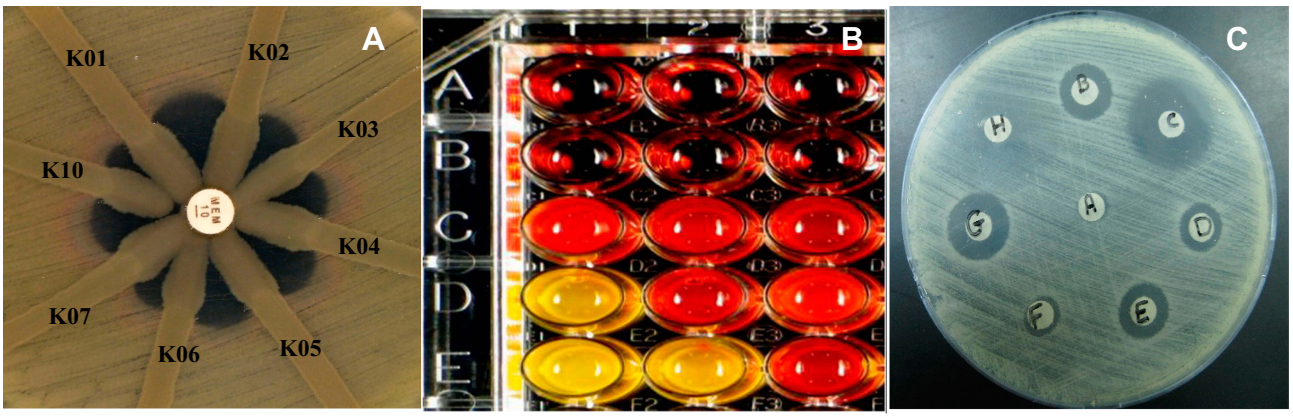

Figure 1 Phenotypic tests for detection and characterization of carbapenemase production. (**A**); Modified Hodge test (K02 to K17 denotes to isolate code), (**B**); Carba NP test (row A; denotes to solution A, row B; denotes to solution B, row C; denotes to solution A containing tested isolates, rows D & E; denote to solution B containing tested isolates, red color indicates negative result, yellow color indicates positive result) (**C**); Combined disk synergy test (disk A; meropenem, disk B; meropenem/clavulanic acid, disk C; meropenem/sulbactam, disk D; meropenem/tazobactam, disk E; meropenem/phenylboronic acid, disk F; meropenem/EDTA, disk G; meropenem/mercaptopropionic acid, disk H; meropenem/sodium chloride).

Abbreviations: NP, Nordmann/Poirel; EDTA, ethylenediaminetetraacetic acid.

(according to colony size) off from an overnight tryptic soy agar (TSA) plate using a sterile yellow tip; the selected colonies were suspended in 500 µL of sterile distilled water, boiled for 10 min, then centrifuged at 13200 rpm for 3 min. The supernatant containing total genomic DNA was transferred to a new 0.5 mL sterile DNase-free tube and stored at −20°C until use.

DNA Fingerprinting

DNA fingerprinting was based on the BOX-PCR technique using the primers and cycling conditions listed in Table 1. BOX-PCR was performed only on chromosomal DNA template. Chromosomal DNA was extracted using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

The following reaction components were transferred into sterile DNase free 0.2 mL PCR tubes (Eppendorf, Germany): 4 µL of DNA template, 4 µL of 5x master mix (Solis BioDyne, Tartu, Estonia), 0.6 µL of forward primer (10 pmol/µL), and 0.6 µL of reverse primer (10 pmol/µL). The volume of the reaction mixture was brought to 20 µL by adding 10.8 µL of sterile DNase-free distilled water. Twelve strains from the laboratory culture collection provided by the microbiology laboratory at Taif University were used as positive controls. *E. coli* ATCC 25922 was used as a negative control strain.

Gene Amplification and Gel Electrophoresis

Gene amplification was performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany), primers from Macrogen (Korea, Geumcheon-gu, Seoul), and the cycling conditions listed in Table 1.

Amplified PCR products corresponding to carbapenemase gene fragments were run on 1% agarose gels containing 0.5 ug/mL ethidium bromide (EtBr), while amplified fragments of the repetitive palindromic sequences of boxA regions were run on 2.5% agarose gels. Agarose gels were run at 100 V for 1.5 hrs using a continuous electrical current power supply (Labnet International Inc, Taiwan).

Extraction and Purification of PCR Products for Gene Sequencing

Amplified bla_{NDM} and bla_{GES} PCR products were cut out of a 1% agarose gel on a UV transilluminator (Bio-Rad, USA) using sterile DNase-free scalpels. Amplified gene fragments entrapped in the gel slices were extracted using the GenElute extraction kit (Sigma-Aldrich, USA). Extracted amplified gene fragments were purified using the ExoSAP-IT One Step PCR Clean-up® Kit (GE Healthcare Limited, USA).

DNA Sequencing

The purified bla_{NDM} and bla_{GES} genes were sequenced by Macrogen using a 3730xl DNA Analyzer (Thermo Fischer Scientific, USA). The primers used for amplification were also used for sequencing.

Sequence Correction and Identification of Query Sequence

Sequencing data were corrected and edited using molecular evolutionary genetic analysis (MEGA) X software (Biodesign Institute, Tempe, USA), as previously described.[29] The corrected bla_{NDM} and bla_{GES} gene sequences were uploaded online to the National Center for Biotechnology and Information (NCBI) and nucleotide basic local alignment search tool (BLASTN) was used to determine the similarity of the query sequences with cited reference sequences.

Phylogenetic Analysis of Corrected of bla_{NDM} Sequence

All corrected bla_{NDM} sequences were phylogenetically analyzed using JalViwe 2.11.0 software[30] in which the genetic relatedness between the identified sequences and the selected reference sequence was determined.

DNA Fingerprint Analysis

The generated DNA fingerprint patterns were analyzed using BioNumeric7.5 software (AppliedMaths, Kortrijk, Belgium), as previously described,[29] in which the phylogenetic relatedness between the investigated isolates was determined by dendrogram construction using Dice (similarity) coefficient with band matching at 1% tolerance and 1.5% tolerance change. Cluster analysis was performed based on the unweighted pair-group method with arithmetic average (UPGMA).

Results

Isolation of CRKP Clinical Isolates

The majority of CRKP clinical isolates were recovered from the Surgical Intensive Care Unit (SICU) with an incidence rate of 26.32% (5/19). On the other hand, 15.79% (3/19) of CRKP isolates were recovered from the urology department

and the Respiratory Intensive Care Unit (RICU). One isolate (8.33%) was recovered from each of the following departments: surgery, cosmetic surgery, hematological diseases, geriatric care, pediatrics, internal medicine, endemic diseases, and ear, nose, and throat (ENT).

Phenotypic Identification and Genotypic Confirmation of CRKP Clinical Isolates

Regarding the phenotypic identification of CRKP clinical isolates, all CRKP isolates were identified as *K. pneumoniae* subspecies *pneumoniae* according to API 20E in which eight different biotypes were identified. Most identified biotypes were related to 5215773, 5205773, and 5005573 biotypes, with frequencies of 42.11% (8/19), 21.05% (4/19), and 10.53% (2/19), respectively.

On the other hand, the lowest identified biotypes were 1205773, 5044552, 5205573, 5215573, and 5214773 where each of which were detected at an incidence of 5.26% (1/19). Regarding genotypic confirmation of biotype clinical isolates, 100% of phenotypically identified isolates tested positive for the *phoE* gene of *K. pneumoniae*.

Susceptibility Testing

AST showed that 19.79% (19/96) of isolates exhibited XDR pattern (data not shown), while none of the isolates exhibited PDR pattern (data not shown). Of the CRKP clinical isolates, 100% were resistant to ampicillin, amoxicillin, cefazolin, cephradine, cefotaxime and cefoperazone, as shown in Table 2. Regarding the other β-lactams that were tested, 5.26% (1/19) of CRKP isolates were susceptible to ceftriaxone, ceftazidime, and aztreonam. Regarding the carbapenems that were tested, 10.53% (2/19) and 5.26% (1/19) of ertapenem non-susceptible isolates exhibited intermediate resistance to imipenem and meropenem, respectively, with regard to non-β-lactam antibiotics, tigecycline and amikacin were the most effective agents, where 94.47% (18/19) and 31.58% (6/19) of CRKP isolates, respectively, were susceptible. Regarding the quinolones that were tested, levofloxacin was more effective than ciprofloxacin; all CRKP isolates were ciprofloxacin-resistant, while 10.53% (2/19) were levofloxacin susceptible.

With regards to β-lactam/β-lactamase combination, the current study showed that clavulanic acid failed to reverse the resistance of amoxicillin and sulbactam also failed to reverse the resistance of both ampicillin and cefoperazone, as shown in Table 2.

Table 2 Antimicrobial Susceptibility Pattern of CRKP Clinical Isolates

Susceptibility Pattern							MIC (μg/mL)	
Antibiotic	Sensitive		Intermediate		Resistant		MIC_{50}	MIC_{90}
	No.	%	No.	%	No.	%		
AM	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
AMS	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
AMX	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
AMC	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
CEZ	–	**0.00**	–	**0.00**	19	**100.00**	1024	>1024
CE	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
CXM	–	**0.00**	–	**0.00**	19	**100.00**	1024	>1024
CTX	–	**0.00**	–	**0.00**	19	**100.00**	1024	>1024
CAZ	1	**5.26**	1	**5.26**	17	**89.48**	>1024	>1024
CPZ	–	**0.00**	–	**0.00**	19	**100.00**	>1024	>1024
CPZS	–	**0.00**	–	**0.00**	19	**100.00**	1024	>1024
CRO	1	**5.26**	–	**0.00**	18	**94.74**	512	>1024
FEP	–	**0.00**	–	**0.00**	19	**100.00**	256	512
ATM	1	**5.26**	–	**0.00**	18	**94.47**	128	1024
IPM	1	**5.26**	2	**10.53**	16	**84.21**	4	16
MEM	–	**0.00**	1	**5.26**	18	**94.74**	16	64
ERT	–	**0.00**	–	**0.00**	19	**100.00**	NA	NA
TET	5	**26.32**	1	**5.26**	13	**68.42**	32	1024
TEG	18	**94.74**	–	**0.00**	1	**5.26**	≤0.5	≤0.5
CN	4	**21.05**	–	**0.00**	15	**78.95**	128	>1024
AK	6	**31.58**	–	**0.00**	13	**68.42**	>1024	>1024
THI	1	**5.26**	–	**0.00**	18	**94.74**	>1024	>1024
CIP	–	**0.00**	1	**5.26**	18	**94.74**	64	128
LEV	2	**10.53**	1	**5.26**	16	**84.21**	32	128

Abbreviations: AM, ampicillin; AMS, ampicillin/sulbactam; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CEZ, cefazolin; CE, cephradine; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CPZ, cefoperazone; CPZS, cefoperazone/sulbactam; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ERT, ertapenem; TET, tetracycline; TEG, tigecycline; CN, gentamicin; AK, amikacin; THI, thiamphenicol CIP, ciprofloxacin; LEV, levofloxacin. Boldface in this table indicates %.

With regards to the tested β-lactams, the MIC_{50} values ranged from 4 to >1024 μg/mL, while the MIC_{90} ranged from 16 to >1024 μg/mL, as shown in Table 2. On the other hand, the lowest detected MIC values corresponded to tigecycline, in which MIC_{50} and MIC_{90} were ≤0.5 μg/mL. With regards to the tested aminoglycosides, the MIC_{50} ranged from 128 to >1024 μg/mL, while the MIC_{90} was >1024 μg/mL. The current study showed that the MIC_{50} values of the tested quinolones ranged from 32 to 64 μg/mL, while the MIC_{90} was 128 μg/mL.

Phenotypic Detection and Characterization of Carbapenemases

The current study revealed that 68.42% (13/19) of CRKP clinical isolates tested positive for MHT and the Carba NP test, as shown in Table 3. On the other hand, phenotypic characterization of class A carbapenemases by CDST

revealed that 42.11% (8/19), 63.16% (12/19), 47.37% (9/19), and 73.68% (14/19) of CRKP isolates responded to the inhibitory effect of clavulanic acid, sulbactam, PBA, and tazobactam, respectively, as shown in Table 4 and Figure 1. On the other hand, the present study revealed that 84.21% (16/19) and 68.42% (13/16) of CRKP isolates tested positive for the inhibitory effect of EDTA and MPA, respectively, as class B carbapenemase inhibitors. Regarding class D carbapenemases, 10.53% (2/19) of CRKP isolates responded to the inhibitory effect of sodium chloride by at least 5 mm. Overall, tazobactam and EDTA were the most effective inhibitors of class A and B carbapenemases, respectively.

Genotypic Detection of Carbapenemases

Regarding class A carbapenemases, the current study showed that bla_{GES} was the only detected gene in which 5.26% (1/19) of CRKP isolates tested positive. Both bla_{KPC} and bla_{SME} were not detected. Regarding class B carbapenemases, 26.32% (5/19) of CRKP clinical isolates tested positive for bla_{NDM}, while bla_{IPM} was not detected. Class D carbapenemases were the most prevalent among CRKP clinical isolates; 73.68% (14/19), 10.53% (2/19), and 21.05% (4/19) of CRKP clinical isolates tested positive for bla_{OXA-48}, bla_{OXA-51}, and $bla_{OXA-181}$, respectively, as shown in Table 5. On the other hand, 5.26% (1/19) of CRKP isolates tested positive for bla_{OXA-23}. None of the isolates tested positive for bla_{OXA-40} and bla_{OXA-58}.

Table 3 Phenotypic and Genotypic Screening Profiles of CRKP Clinical Isolates

Isolate No. Carbapenemase Profile		
	Phenotypic	**Genotypic**
KP01	MHT, CNP	bla_{OXA-48}
KP02	-	bla_{OXA-48},bla_{OXA181}
KP03	MHT	bla_{GES}, bla_{NDM},$bla_{OXA-23}$$bla_{OXA-48}$, bla_{OXA-51}, bla_{OXA181}
KP05	MHT	bla_{OXA-48}
KP06	MHT	bla_{OXA-48}
KP07	CNP	bla_{OXA-48}
KP08	MHT	bla_{OXA-48}, bla_{OXA181}
KP09	CNP	bla_{OXA-48}
KP12	MHT, CNP	bla_{OXA-48}
KP13	CNP	bla_{OXA-48}
KP14	CNP	bla_{NDM}, bla_{OXA-48}
KP16	MHT, CNP	bla_{NDM}, bla_{OXA-48}, bla_{OXA181}
KP17	MHT, CNP	bla_{OXA-48}
KP18	MHT, CNP	bla_{OXA-51}
KP19	MHT, CNP	bla_{NDM}
KP21	MHT, CNP	-
KP22	MHT, CNP	bla_{NDM}
KP23	MHT	bla_{OXA-48}
KP24	CNP	-

Abbreviations: MHT, modified Hodge test; CNP, Carba Nordmann/Poirel; CRKP, carbapenem-resistant *K. pneumoniae*; KP, *Klebsiella pneumoniae* isolate code No.

DNA Sequencing

Two allelic variants of bla_{NDM} were detected as shown in Figure 2; 40% (2/5) are related to bla_{NDM-1} and 60% (3/5) are related to bla_{NDM-25}. On the other hand, bla_{GES-1} was the detected variant of bla_{GES}, which has ESBL activity but not carbapenemase activity.

DNA Fingerprint and Cluster Analysis

Phylogenetic analysis of the banding pattern generated by Box-PCR (Figure 3) revealed 18 different profiles in which two isolates (K12 and K13) exhibited a single identical profile, whereas the other 17 isolates exhibited 17 different profiles. The generated UPGMA dendrogram classified CRKP isolates into two phylogenetic groups (A&B), as shown in Figure 4. Phylogenetic group A included only one isolate (KP19) that was recovered from the RICU. The genetic profiles of the remaining isolates belonged to phylogenetic group B. Phylogenetic group B was further classified into two main clusters (B1 and B2). Cluster B1 included only one isolate (KP2) that was recovered from the pediatrics department. All the remaining isolates were grouped into the B2 cluster that was further classified into three clades (B2.A, B2.b1, and B2b2). Overall, 73.68% (14/19) of the CRKP isolates were classified into the B2.b2 clade.

Discussion

K. pneumoniae is one of the most problematic bacterial pathogens and commonly exhibits the MDR pattern. *K. pneumoniae* is associated with increased mortality and morbidity of hospitalized patients, especially in acute care settings like intensive care units (ICUs) and cardiac care units (CCUs).[31]

Carbapenems are one of the most effective agents for treating infections caused by MDR *K. pneumoniae*.[32] Unfortunately, resistance to carbapenem among *K. pneumoniae* continues to increase. Data released by the CDC indicated that 8% of *Klebsiella* spp. are carbapenem-resistant.[33]

To the best of our knowledge of the cited literature, the issue of carbapenem resistance in Egypt continues to worsen, which is alarming and represents a serious problem that may be encountered in the future. The current study was designed to investigate the rate and genetic background of carbapenem resistance in *Klebsiella* spp. clinical

Table 4 Carbapenemase Inhibitor Profile of CRKP Clinical Isolates

Carbapenemase Inhibitor Profile								
Isolate No.	**Class A Inhibitors**				**Class A and Specific bla_{KPC} Inhibitor**	**Class B Inhibitor**		**Class D Inhibitor**
	CA	**Sul**	**Taz**	**PBA**	**PBA**	**EDTA**	**MPA**	**NaCl**
KP01	+	+	+	–	+	+	+	–
KP02	–	+	+	–	–	+	+	–
KP03	+	+	+	–	+	+	+	–
KP05	–	–	–	–	+	–	+	–
KP06	–	–	–	–	–	–	+	–
KP07	+	+	+	–	+	+	+	–
KP08	+	+	+	–	+	+	+	+
KP09	+	+	+	–	–	+	–	–
KP12	–	–	–	–	–	+	–	–
KP13	–	–	–	–	–	+	–	–
KP14	+	+	+	–	+	+	+	–
KP16	–	–	–	–	–	+	–	–
KP17	–	+	+	–	–	+	–	–
KP18	+	+	+	–	+	+	+	–
KP19	–	–	+	–	+	+	+	–
KP21	–	+	+	–	–	+	+	+
KP22	+	+	+	–	–	+	–	–
KP23	–	–	+	–	–	–	+	–
KP24	–	+	+	–	+	+	+	–

Abbreviations: CA, clavulanic acid; Sul, sulbactam; Taz, tazobactam; phenyl boronic acid; EDTA, ethylenediaminetetraacetic acid; MPA, mercaptopropionic acid; NaCl, sodium chloride; PBA, phenyl boronic acid; CRKP, carbapenem resistant *K. pneumoniae*; KP, *Klebsiella pneumoniae* isolate code No.

isolates recovered from a large tertiary care hospital in Cairo, Egypt. The results of the current study showed that the rate of carbapenem resistance was 19.79%, in which 19 out of 96 *Klebsiella* spp. were resistant to either imipenem, ertapenem, and/or meropenem.

Our findings are consistent with the findings of Wiener-Well et al[34] who reported that the rate of carbapenem resistance in *Klebsiella* spp. ranged from 8% to 25%.[34] Both the findings of the present study and that of Wiener-Well et al[34] are far from the findings of the recent Egyptian[35] and Iranian[36] studies which reported that carbapenem resistance rates among *K. pneumoniae* clinical isolates were 44.4% and 63.8%, respectively.

Fortunately, the present study did not find any isolates exhibiting the PDR pattern which is consistent with the finding of Wenzi Bi et al[37] who reported that no *K. pneumoniae* isolates exhibited the PDR pattern where all XDR *K. pneumoniae* isolates were susceptible to tigecycline.

The dissemination of carbapenemase-producing Gram-negative isolates inside hospitals is a major health concern. Early and rapid detection of these isolates is crucial for initiating effective therapeutic options as early as possible in order to achieve desirable outcomes, especially for serious infections and to prevent the spread of these XDR isolates,[21,22] based on that, the current study used three phenotypic methods for detection of carbapenemase production among CRKP clinical isolates: (i) MHT (ii), Carb NP test, and (iii) CDST.

Although Carba NP is a cost-effective and rapid test for detecting carbapenemase production, this test in the current study seems to not be highly sensitive, based on the test's failure to detect carbapenemase production by six isolates that were genotypically confirmed to harbor at least one carbapenemase gene, including OTCs and MBLs, this conclusive finding is identical to the previously reported limitations of the Carba NP test declared by the CLSI.[38] Similar reports of low sensitivity for the Carba NP test have been reported.[21,22,39]

In contrast to the Carba NP test that failed to detect six isolates that were genotypically confirmed to carry at least

Table 5 Detailed Resistance and Genetic Profiles of CRKP Clinical Isolates

Isolate No.	Antibiotic Resistance Profile	No. of Resistant Antibiotics	Detected Carbapenemases	No. of Detected Carbapenemases
KP01	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, FEP, IPM, MEM, ERT, CN, AK, CIP	18	bla_{OXA-48}	1
KP02	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{OXA-48}, bla_{OXA181}	2
KP03	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{GES},bla_{NDM}, bla_{OXA-23}, bla_{OXA-48}, bla_{OXA-51}, bla_{OXA181}	6
KP05	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, MEM, ERT, TET, CN, THI, CIP, LEV	21	bla_{OXA-48}	1
KP06	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, THI, CIP, LEV	20	bla_{OXA-48}	1
KP07	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, THI, CIP, LEV	22	bla_{OXA-48}	1
KP08	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	22	bla_{OXA-48},bla_{OXA181}	2
KP09	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, TEG, CN, AK, THI, CIP, LEV	24	bla_{OXA-48}	1
KP12	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{OXA-48}	1
KP13	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{OXA-48}	1
KP14	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{NDM}, bla_{OXA-48}	2
KP16	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, CN, AK, THI, CIP, LEV	22	bla_{NDM}, bla_{OXA-48}, bla_{OXA181}	3
KP17	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, CN, AK, THI, CIP, LEV	22	bla_{OXA-48}	1
KP18	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, THI, CIP, LEV	21	bla_{OXA-51}	1
KP19	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, THI, CIP, LEV	21	bla_{NDM}	1
KP21	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	-	-
KP22	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, THI, CIP	20	bla_{NDM}	1
KP23	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, TET, CN, AK, THI, CIP, LEV	23	bla_{OXA-48}	1
KP24	AMP, SAM, AMX, AMC, CEZ, CE, CXM, CTX, CAZ, CPZ, CPS, CRO, FEP, ATM, IPM, MEM, ERT, CN, AK, THI, CIP, LEV	22	-	-

Abbreviations: AMP, ampicillin; SAM, ampicillin/sulbactam; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CEZ, cefazolin; CE, cephradine; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CPZ, cefoperazone; CPS, cefoperazone/sulbactam; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ERT, ertapenem; TET, tetracycline; TEG, tigecycline; CN, gentamicin; AK, amikacin; THI, thiamphenicol; CIP, ciprofloxacin, LEV, levofloxacin; CRKP, carbapenem resistant; *K, pneumoniae*, KP, *Klebsiella pneumoniae* isolate code No.

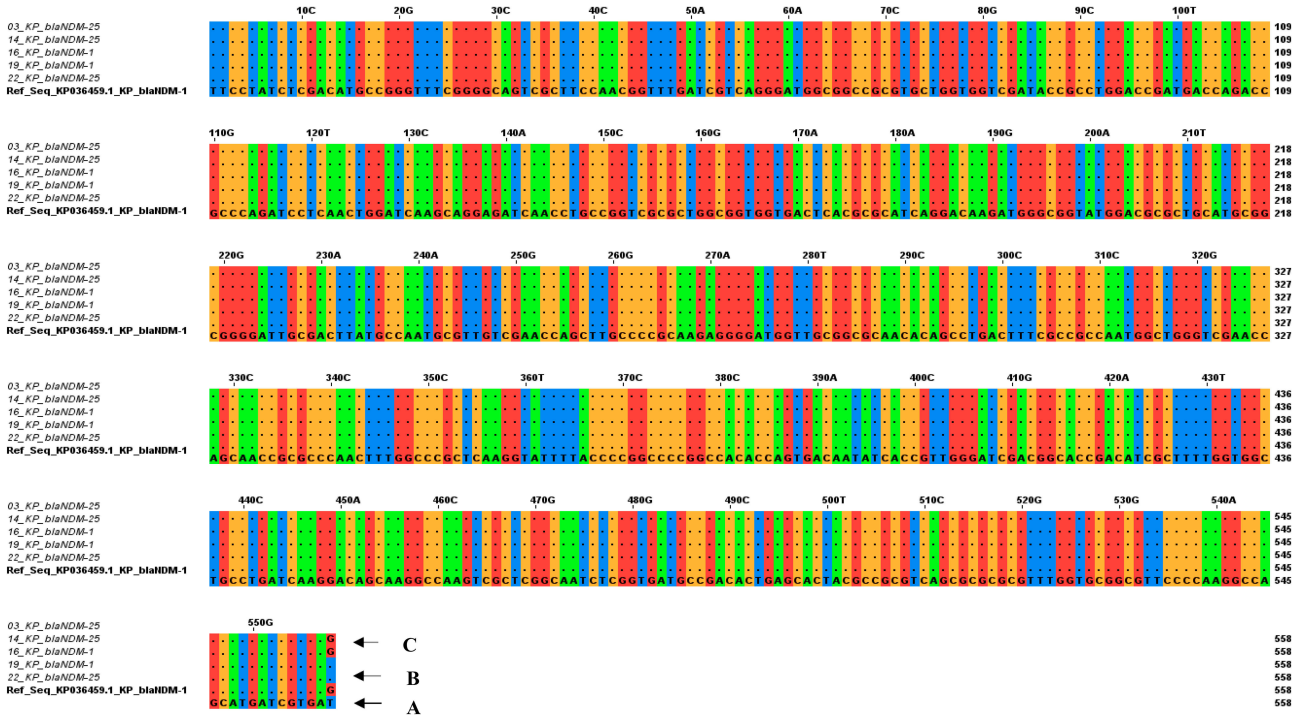

Figure 2 Multiple sequence alignment of sequenced *bla*$_{NDM}$ with the reference sequence *bla*$_{NDM-1}$ KP036459.1; the arrow (**A**) is pointing to thymine nucleotide in reference sequence *bla*$_{NDM-1}$ KP036459.1; the arrow (**B**) is pointing to non-mutated thymine nucleotide at position 558 in the query sequence of investigated *bla*$_{NDM-1}$; the arrow (**C**) is pointing to mutated guanine nucleotide at position 558 in the query sequence of investigated *bla*$_{NDM-25}$.

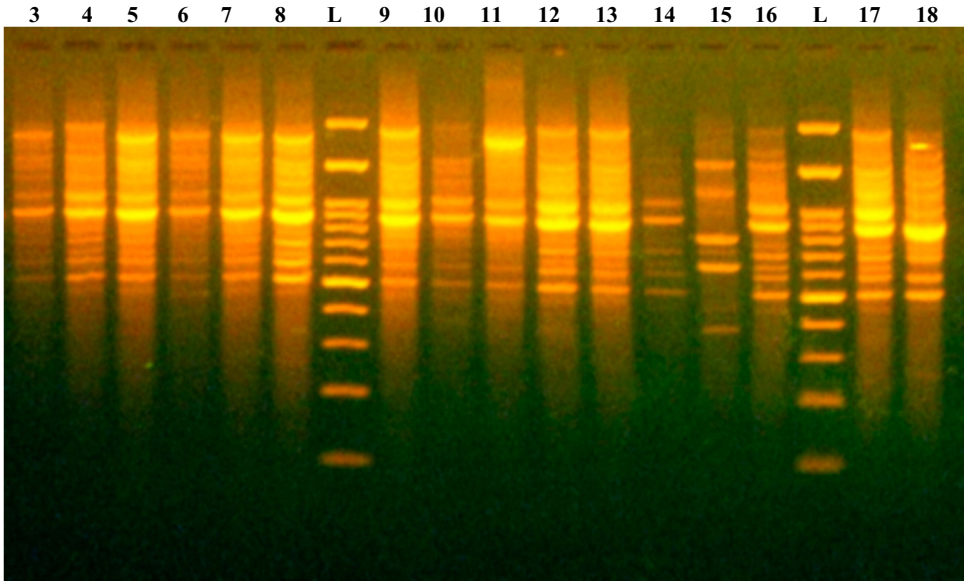

Figure 3 Representative DNA fingerprint pattern generated by BOX-PCR for carbapenem-resistant *K. pneumoniae* clinical isolates. L, 100 bp DNA ladder; lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18 are the code No. of CRKP clinical isolates.

one carbapenemase gene, the present study revealed that MHT was more sensitive than the Carba NP test, based on failure of MHT to detect only five isolates that were genotypically confirmed to harbor at least one carbapenemase gene.

The current study revealed the superiority of MHT over Carba NP test for detection of carbapenemases, this finding was also reported by a recent Egyptian study[35] that showed a high sensitivity for MHT in which 100% (14/14) of CRKP isolates, that were molecularly confirmed to be

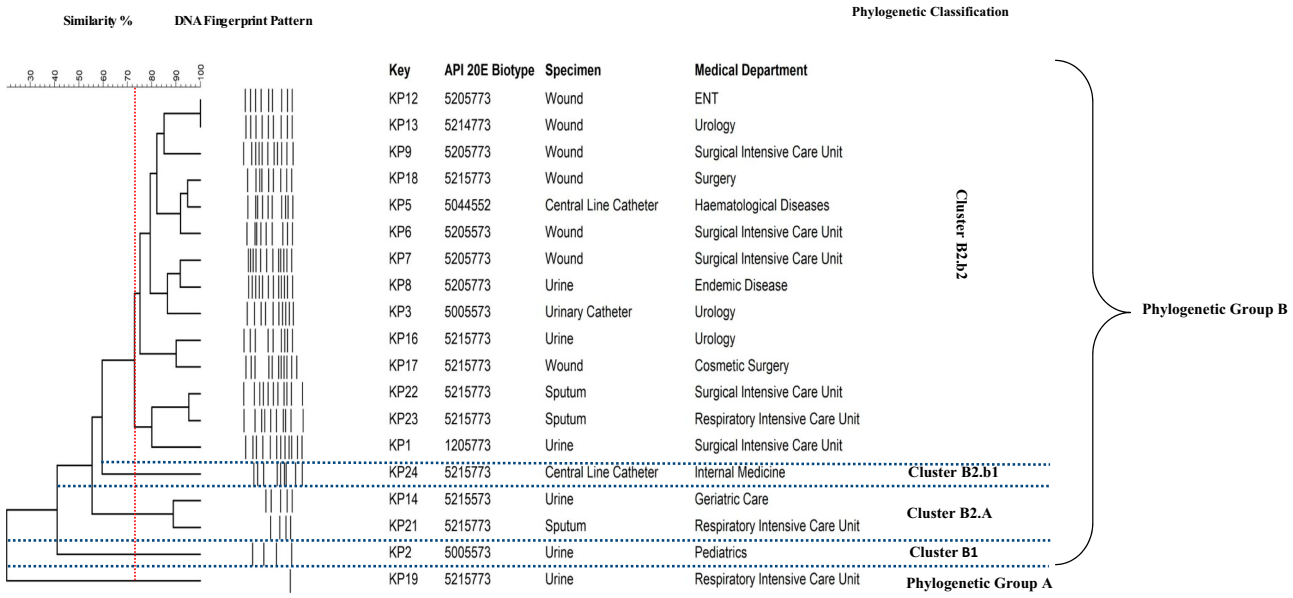

Figure 4 Unweighted pair-group method with arithmetic average (UPGMA) dendrogram based on Dice similarity for banding profile generated by BOX-PCR.

carbapenemase producers, were tested positive for MHT. Similar findings were reported by AlTamimi et al[40] who found that the sensitivity of MHT among CRKP isolates was 92% (23/25). In the current study, we supposed that the negative MHT result for the five isolates that genotypically tested positive for carbapenemases may be due to false negatives rather than true negatives. Similar conclusive false-negative results for MHT were reported by the CLSI.[38] The present study suggests that the positive MHT result of the one isolate that tested negative for all investigated carbapenemase genes may be due to (i) false-positive results due to ESBL production or AmpC production coupled with porin loss[38] or (ii) the presence of other non-investigated carbapenemase genes.

In contrast to our conclusive findings regarding the higher sensitivity of MHT over Carba NP test, Bayramoğlu et al[41] reported that the Carba NP test is more sensitive than MHT.

With regards to the negative results of CDST that was used to characterize carbapenemases in genotypically confirmed isolates that harbor carbapenemases genes, the present study suggests that the negative result may be attributed to one or more of the following four possibilities: (i) low level of expression of the carbapenemase gene(s), (ii) the presence of non-expressed carbapenemase gene(s), (iii) loss of a plasmid that carries the detected carbapenemase gene(s) during the testing period, and (iv) the presence of other resistance mechanisms in addition to the detected carbapenemases.

Regarding the phenotypic characterization of carbapenemases using PBA acid as class A carbapenemase and KPC inhibitor, the current study showed that 47.37% (9/19) of CRKP isolates tested positive, although none of the isolates tested positive for bla_{KPC}, indicating that these results may be false positives due to AmpC hyperproduction[42] or the presence of other class A carbapenemase genes that were not investigated in this study.

Although carbapenemase production can be detected by phenotypic tests, molecular detection of these enzymes remains the gold standard.[22] The current study revealed that OTCs were the most prevalent carbapenemases that detected in the investigated isolates, in which 78.95% (15/19) of CRKP isolates were found to harbor at least one of the OCTs.

Of the detected OTCs, OXA-48 was the most prevalent one in which 73.68% (14/19) of isolates were found to harbor bla_{OXA-48}. Similar findings were reported by the Turkish,[43] Egyptian,[31] and Saudi[40,44] studies that reported that bla_{OXA-48} was more prevalent in CRKP clinical isolates.

To the best of our knowledge of the cited literature, few Egyptian studies have investigated the prevalence of bla_{NDM} in CRKP isolates; thus, the current study investigated this issue. The present study detected only five isolates that harbor bla_{NDM}, corresponding to a rate of 26.32%, this finding is somewhat closely related to a recent Egyptian study[31] that reported only two CRKP isolates were found to harbor bla_{NDM} at a rate of 4.3%.

Although several reports have been published by some Asian countries like India[45] and neighboring countries

including Pakistan,[46] Bangladesh,[47] and Sri Lanka[48] indicating the existence of $bla_{OXA-181}$ in CRKP, there are no Egyptian reports indicating the detection of $bla_{OXA-181}$ or the coexistence of $bla_{OXA-181}$ and bla_{NDM} in CRKP clinical isolates.

Based on the above, the current study investigated the existence of $bla_{OXA-181}$ in CRKP and addressed the first report for the coexistence of $bla_{OXA-181}$ and bla_{NDM} in CRKP clinical isolates recovered from Egyptian patients. Similar reports have been published by other African countries like Nigeria and Angola.[49]

With regards to bla_{OXA-23}, many Egyptian reports[50–52], have documented the existence of such gene in carbapenem-resistant *Acinetobacter baumannii* clinical isolates rather than in CRKP clinical isolates.

Based on our knowledge of the cited literature, the current study documents the first African report for the detection of bla_{OXA-23} in CRKP clinical isolates recovered from Egyptian patients, this finding was confirmed by reviewing the African studies that have investigated such topic and it was found that only one study in this regard has been done in Algeria[53] in which bla_{OXA-23} was detected only in two *Acinetobacter baumannii* clinical isolates and was not detected in *K. pneumoniae* clinical isolates.

Although bla_{OXA-23} bla_{OXA-40}, and bla_{OXA-58} are plasmid encoded genes that were detected mainly in *A. baumannii*, while bla_{OXA-51} is mainly a chromosomal gene in *A. baumannii*,[54] the current study has investigated the four previously mentioned genes in CRKP clinical isolates to check the ability of these genes to transfer from *A. baumannii* to *K. pneumoniae* clinical isolates.

As mentioned before, bla_{OXA-23} bla_{OXA-40}, and bla_{OXA-58} are mainly plasmid-encoded genes so, they have the chance to transfer to other bacterial genera by conjugation.[54] On the other hand, gene transposition from bacterial chromosome to plasmid is well documented in *A. baumannii* so, $bla_{OXA-511}$ can be transferred from the chromosome to plasmid in *A. baumannii*, and hence, plasmid can be transferred from *A. baumannii* to *K. pneumoniae*.[55] It was documented that bla_{OXA-51} was detected in *K. pneumoniae* through the case report conducted by Budak S et al.[56]

Although bla_{SME} is a carbapenemase gene the carried mainly on the chromosome of *Serratia marcescens*, it was reported that such gene can be disseminated to other enterobacterial pathogens based on the study that conducted by Lee CR et al[57] so, the current study investigated the existence of this gene in CRKP clinical isolates and it was found that no isolate harbored bla_{SME}.

With regards to bla_{GES} that was detected in only one isolate (KP03), it was found that this gene had no carbapenemase activity in which DNA sequencing results revealed that the detected variant was bla_{GES-1} which is well known to have only extended-spectrum β-lactamases (ESBL). Interestingly, bla_{GES} was not detected in *K. pneumoniae* clinical isolates in Egypt, but instead it was detected in other Egyptian Gram-negative isolates like *Acinetobacter baumannii*[51] and *E. coli*.[16]

The BOX-PCR fingerprint profile indicated that, although CRKP clinical isolates were recovered from different patients in different hospital departments, the circulating *K. pneumoniae* isolates in this hospital are highly genetically related, where 18 isolates belong to a single phylogenetic group (B).

A limitation of the present study was the limited number of carbapenem-resistant isolates that were tested for the 12 carbapenemase genes; thus, additional studies will be done to further document the prevalence of $bla_{OXA-181}$ and bla_{OXA-23} in CRKP clinical isolates.

In conclusion, the current study is the first Egyptian report that addressed the coexistence of bla_{NDM-25}, bla_{OXA-23}, $bla_{OXA-181}$, and bla_{GES-1} in CRKP clinical isolates that are genetically related based on DNA fingerprint profiles obtained by BOX-PCR. Also, the current study addressed the limitations of MHT and Carba NP test for the detection of carbapenemases in isolates that tested positive for bla_{NDM} and OTCs.

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Disclosure

The authors declare no conflict of interest in this work.

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