


Molecular Characterization of Carbapenemase-Producing *Klebsiella pneumoniae* Isolated from Egyptian Pediatric Cancer Patients Including a Strain with a Rare Gene-Combination of β -Lactamases

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Purpose: Healthcare-associated infections caused by multi-drug-resistant (MDR) pathogens are a global threat. We aim to assess the clonal relatedness among carbapenemase-producing *Klebsiella pneumoniae* (CPKP) strains infecting Egyptian pediatric cancer patients.

Materials and Methods: Identification and antimicrobial susceptibility testing of 149 Gram-negative isolates obtained from pediatric cancer patients were performed by VITEK 2. Genes encoding carbapenemases and extended-spectrum β -lactamases were detected by PCR and verified by DNA sequencing of representative samples. The transferability of the plasmids harboring *bla*_{OXA-48}, from representative clinical samples, was evaluated by performing a conjugation experiment followed by PCR and MIC shift determination. Clonal relationships among the *bla*_{OXA-48}-harboring *K. pneumoniae* isolates were determined by enterobacterial repetitive intergenic consensus (ERIC)-PCR and pulsed-field gel electrophoresis (PFGE).

Results: Carbapenem resistance was observed in 59% of the isolates. The most prevalent species was *K. pneumoniae* (45.6%) and 57% of them were isolated from ICU. Fifty-nine % of the *K. pneumoniae* isolates were carbapenemase-producers and *bla*_{OXA-48} was detected in (58%) of them. One isolate co-harbored *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP-1} genes for the first time in Egypt. PCR and meropenem MIC shift confirmed the success of the transferability of representative plasmids to *E. coli* K12. ERIC and PFGE identified 93% and 100% of the *K. pneumoniae* with a similarity coefficient $\geq 85\%$, respectively, including strains with indistinguishable patterns, suggesting possible clonal dissemination.

Conclusion: Our findings underline the dissemination of diverse clones of MDR CPKP among Egyptian pediatric cancer patients. Hence, routine molecular characterizations followed by strict implementation of infection control measures are crucial to tackling this threat.

Keywords: ERIC-PCR, PFGE, *bla*_{OXA-48}, clonal dissemination, Egypt, carbapenem-resistant

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Introduction

Bacterial infections have been implicated as one of the most frequent complications that threaten immunosuppressed cancer patients. As those patients are frequently hospitalized for treatment purposes.^{1,2} Gram-negative bacteria such as

Enterobacteriaceae, mainly *K. pneumoniae* and *E. coli* followed by *Acinetobacter* and *Pseudomonas*, are commonly reported as infecting pathogens in cancer patients.^{3,4} In 2019, the CDC listed carbapenem-resistant Enterobacteriaceae (CRE) as an urgent threat.⁵ Of special concern, the emergence of carbapenem-producing *K. pneumoniae* (CPKP) which is responsible for hospital-acquired infection (HAI) outbreaks in cancer wards that became the main reason for the increased rates of mortality among the hospital settings.⁶

The spread of Enterobacteriaceae is attributed to their ability in carrying resistance plasmids coding for enzymes that interfere with important antibiotics used as empiric therapy in febrile neutropenic patients; cephalosporins and carbapenems.⁷ Particularly, those that produce carbapenemase enzymes including, *K. pneumoniae* carbapenemases (KPCs), Metallo- β -lactamases (MBL), and oxacillinase (OXA-48). Carbapenemases are commonly categorized using the Ambler classification scheme. KPCs are the most common carbapenemases identified, and they are members of class A. They are commonly found in Enterobacteriaceae and can be occasionally identified in *P. aeruginosa* or *A. baumannii*. Class B Metallo β -lactamases (MBLs), commonly include (NDM, VIM, and IMP) enzymes. In addition, class D carbapenemases include OXA-48-like enzymes, which are generally produced by Enterobacteriaceae, while other variants are frequently produced by *A. baumannii*. Other mechanisms that may contribute to carbapenem resistance in Enterobacteriaceae include; reduced uptake of β -lactam drugs by selectively altering the cell membrane porin channels, decreased membrane permeability, efflux pumps, and the presence of various broad-spectrum β -lactamases (eg, ESBL and/or AmpC cephalosporinase).^{8–10}

Carbapenem resistance is considered a great threat as they are usually combined with other resistance mechanisms for a wide range of antimicrobial agents; thus, infections with carbapenemase-producing Enterobacteriaceae are associated with high mortality rates. Hospital-acquired infection outbreaks are also associated with Enterobacteriaceae producing extended-spectrum β -lactamases (ESBLs) that have been identified as a major cause of the MDR phenotype, because of their wide diversity of enzymes, including TEM, SHV, and CTX-M.^{3,7,11,12} Investigation of infection outbreaks, the transmission of microorganisms between the environment and the patients, and epidemiological surveillance have become an important

tool in monitoring local dissemination of resistant bacterial strains within the healthcare facilities.^{1,13,14} Accordingly, phenotypic methods have been replaced by molecular typing methods that examine the relatedness of microorganisms at the genomic level.¹⁵ To date, few studies have focused on carbapenem resistance in pediatric patients, especially in critically ill children.¹⁶ Accordingly, in this study, the clonality of CPKP strains causing infections in pediatric cancer patients was investigated using two different molecular typing methods; Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR and pulsed-field gel electrophoresis (PFGE).

Methods

Bacterial Strains and Microbiological Methods

The epidemiology of carbapenem-resistant Enterobacteriaceae was studied in a 320-bed tertiary care hospital (Children's Cancer Hospital) for pediatric cancer patients between November 2014 and August 2016, in Cairo, Egypt. A total of 149 non-duplicated Gram-negative isolates were collected. All isolates were identified by both biochemical testing and VITEK 2 automated system using the ID-GNB card for identification of Gram-negative bacilli according to the manufacturer's instructions (bioMérieux, France). The study approval was obtained from the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (approval no. MI-1203). No patients' consent form was required as all the isolates were obtained from the hospital microbiology lab after being grown on MacConkey and Trypticase Soy Agar, for routine hospital work, then stored at -80°C in LB medium supplemented with 15% v/v glycerol.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing to a panel of antibiotics according to the hospital routine was performed using the Kirby Bauer disc diffusion method.¹⁷ Minimum inhibitory concentration (MIC) for amikacin, cefepime, cefoxitin, cefotaxime, ceftriaxone, gentamicin, ciprofloxacin, tobramycin, meropenem, and piperacillin-tazobactam were determined by the VITEK 2 system using the AST-GN card (bioMérieux) and confirmed for meropenem, imipenem, and colistin using the E-test strips (bioMérieux). The interpretation was according to the CLSI breakpoint values.¹⁸ *E. coli* ATCC 25,922 and

ATCC 35,218 were used as negative and positive quality control strains for susceptibility testing, respectively.¹⁹

Phenotypic Detection of Carbapenemases

According to the CLSI recommendations, Modified Hodge Test (MHT), Carba NP (RAPIDEC, bioMérieux), and the modified Carbapenem Inactivation Method (mCIM) tests were used to assess the ability of the carbapenem-resistant isolates to produce carbapenemases.^{18,20,21} For the MHT

K. pneumoniae ATCC BAA-1705 and ATCC BAA-1706 were used as a positive and negative control, respectively. While, *K. pneumoniae* ATCC 700,603 was used as a positive control for ESBL.¹⁹

Molecular Analysis of Antibiotic Resistance Determinants

Total DNA was extracted using the boiling method.²² An in-house PCR test for carbapenemase-encoding genes was performed using specific primers for (*bla*_{KPC}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{OXA-48}, and *bla*_{IMP}), as well as the ESBLs

(*bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}). Briefly, PCR reactions were carried out in a total volume of 25 µL reaction mixture containing 1 µL (~50 ng/mL) bacterial DNA template, 12.5 µL DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific), and 10 µM (1 µL) from each primer, and nuclease-free water to complete the reaction to 25 µL. PCR cycling conditions have been optimized at different annealing temperatures (Ta) for different primer sets (Table 1). PCR reactions were carried out using a gradient PCR thermocycler (Analytik, Jena, Germany). A reaction with no DNA-template was used as a negative control, while *K. pneumoniae* ATCC 700,603 was used as ESBL-positive control.¹⁹ In order to verify that the obtained PCR products are indeed the intended gene fragments, representative samples of the PCR products were subjected to DNA sequencing analyses. The FinchTV software was used to analyze the sequencing data. The DNA sequence of each gene fragment was compared to the sequences in the GenBank nucleotide database using the BLAST tool (<http://www.ncbi.nlm.nih.gov/blast/>).²³

Table 1 List of Primers Used in the Current Study

Class	Target Gene	Sequence	Amplicon Size (bp)	Annealing Temperature (Ta)	Reference
Class A	<i>bla</i> _{KPC}	F 5'-GCGGAACCATTCGCTAAACT-3' R 5'-GGCGGCGTTACTACTGTATT-3'	312	55°C	This study
Class B	<i>bla</i> _{NDM}	F 5'-GTTTGATCGTCAGGGATGGC-3' R 5'-CCGTATGAGTGATTGCGGC-3'	558	55°C	
	<i>bla</i> _{IMP}	F 5'-GAAGAAGTTAACGGGTGGGG-3' R 5'-GCATACGTGGGGATGGATTG-3'	227	52°C	
	<i>bla</i> _{VIM}	F 5'-GCAAGTCCGTTAGCCCATTC-3' R 5'-CACCCCACGCTGTATCAATC-3'	208	53°C	
Class D	<i>bla</i> _{OXA-48}	F 5'-GGGCGTGGTTAAGGATGAAC-3' R 5'-CATCTTGCTCATACGTGCCT-3'	166	55°C	This study
ESBLs	<i>bla</i> _{CTX-M}	F 5'-TTTGCGATGTGCAGTACCAGTAA-3' R 5'-CGATATCGTTGGTGGTGCCATA-3'	544	55°C	
	<i>bla</i> _{SHV}	F 5'-TGGTTATGCGTTATATTCGCC-3' R 5'-GGTTAGGGTTGCCAGTGCT-3'	868	54°C	
	<i>bla</i> _{TEM}	F 5'-TCGCCGCATACACTATTCTCAGAATGA-3' R 5'-ACGCTCACCGGCTCCAGATTAT-3'	445	59°C	
ERIC primers	1- (5'-ATGTAAGCTCCTGGGGATTAC-3') 2- (5'-AAGTAAGTACTGGGGTGAGCG-3')		45°C	62	

Molecular Typing

ERIC Typing

Selected *K. pneumoniae* isolates that harbor the *bla*_{OXA-48} gene were analyzed for typing purposes using the ERIC-PCR. Genomic DNA was extracted by the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The PCR reaction contained 12.5 µL of DreamTaq Green PCR Master Mix (2X), 50 ng of bacterial DNA template, 2 µL from the ERIC primers (Table 1), and nuclease-free water to complete the total volume to 25 µL. According to the protocol described by Bilung and colleagues²⁴ with slight modifications, the conditions used were as follows: initial denaturation for 15 min at 95°C followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 45°C, the extension for 8 min at 72°C, and a final extension for 15 min at 72°C.

ERIC-PCR fingerprint was analyzed using BioNumerics 7.6 software (Applied Maths, Belgium), based on the Dice similarity coefficient and using the unweighted pair group method with arithmetic mean (UPGMA).²⁵ Band position tolerance was 1.5%, and the optimization was 1%. Isolates with a similarity coefficient $\geq 85\%$ were considered to belong to the same cluster.

PFGE Typing

Pulsed-field gel electrophoresis (PFGE) was performed using 50 U XbaI restriction enzyme (NEB, USA). Macro-restriction of genomic DNA was carried out to establish clonal relationships between *K. pneumoniae* resistant isolates, according to the PulseNet PFGE protocol from the Centers for Disease Control and Prevention (CDC) with minor modifications.²⁶ Electrophoresis was performed on a 1% low-melting agarose gel (Sigma-Aldrich, USA) using a CHEF DR II apparatus (Bio-Rad, USA). PFGE conditions were as follows: pulse times ranged from 5 to 40s over 18 h at 6.0 V/cm and at 14°C. DNA fragment patterns interpretation was carried out according to the Tenover criteria.²⁷ DNA fragment patterns were normalized using the bacteriophage Lambda PFG ladder (NEB). A dendrogram was generated based on the dice coefficient determination, using the UPGMA with 1.5% band matching tolerance and optimization in BioNumerics software version 7.6.

Conjugation Experiments

To determine the transferability of the *bla*_{OXA-48} gene, broth mating assay was implemented using the Luria-

Bertani (LB) broth medium, *E. coli* K12 served as the recipient strain and two clinical isolates harboring the *bla*_{OXA-48} gene served as the donor cells as previously described.²⁸⁻³⁰ Transconjugants were selected on MacConkey agar plates containing ampicillin (100 µg/mL) and meropenem (50 µg/mL). The success of acquiring the resistance plasmid was tested by performing a PCR on the *E. coli* K12 before and after conjugation. In addition, the MIC of meropenem for the transconjugants was compared to the donors and *E. coli* K12 to further confirm the transferability of the resistance gene. The experiment was performed using two *K. pneumoniae* strains as donors as they exhibited the highest MIC value towards meropenem (isolates 116 and 128) in the collected isolates.

Statistical Analyses

Statistical analyses were carried out using the software package SPSS[®] v23 (SPSS Inc., USA). Categorical variables were described using the Chi-square test. Interquartile range and mean were used for continuous variables, according to data distribution. A *P*-value of <0.05 was considered significant. The genetic diversity and discriminatory index (DI) for the typing techniques for both ERIC and PFGE were calculated using Hunter and Gaston's formula.³¹ DI values range from 0 to 1; the least and the most possible biodiversity in the population.

Results

Clinical and Epidemiological Characteristics

During the study period, a total of 149 non-duplicated Enterobacteriaceae isolates were obtained from various clinical specimens. The strains were isolated from 140 pediatric cancer patients, with an age range (2 weeks–17 years, median 6 months). The majority of the patients were males 81 (58%). *K. pneumoniae* *n*=68 (45.6%), was the most prevalent species. Accordingly, these isolates would be the focus of the current study. The distribution of the *K. pneumoniae* isolates among gender, different clinical specimens, and wards is shown in (Table 2).

Antimicrobial Susceptibility

According to the MIC test results of all the collected isolated, 139 isolates were considered MDR as they were non-susceptible to three or more antimicrobial classes.³²

Table 2 Relationship Between Different Carbapenemases and β -Lactamases Among the *Klebsiella pneumoniae* Isolates and Patient's Clinical Data

Characteristics	Total	bla _{oxA-48}	p-value	bla _{NDM}	p-value	bla _{VIM}	p-value	bla _{IMP}	p-value	bla _{SHV}	p-value	bla _{TEM}	p-value	bla _{CTX-M}	p-value
Gender															
Male	38	16	0.7	5	0.53	1	0.37	1	0.86	11	0.2	16	0.86	14	0.76
Female	30	14		7		0		1		5		12		10	
Ward															
ICU	39	21	0.32	4	0.01 ^a	1	0.9	2	0.92	11	0.7	10	0.02 ^a	16	0.6
Haematology	16	4		5		0		0		3		9		5	
Solid tumour	3	1		3		0		0		1		3		1	
Surgery	6	3		0		0		0		1		4		2	
Stepdown	4	1		0		0		0		0		2		0	
Specimen															
Blood	40	18	0.1	5	0.94	1	0.98	1	0.92	13	0.35	15	0.46	16	0.32
CVP	17	5		4		0		1		3		7		6	
Urine	2	0		1		0		0		0		2		0	
CSF	1	0		0		0		0		0		1		0	
BAL	3	3		1		0		0		0		1		2	
Wound	5	4		1		0		0		0		2		0	

Note: ^aValues with $p \leq 0.05$ are considered significantly different.

Abbreviations: CVP, central venous portai; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid.

Among those MDR isolates, 88 were classified as carbapenem-resistant Enterobacteriaceae (CRE). The majority of the CRE isolates were *K. pneumoniae* (59%, n=52). Overall, the collected *K. pneumoniae* isolates showed diminished susceptibility for the tested antibiotics; meropenem, imipenem, ampicillin, piperacillin/tazobactam, ceftazidime, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, gentamicin, and tobramycin. However, marked susceptibility for amikacin, tigecycline, and colistin was observed as illustrated in (Figure 1).

Phenotypic Detection of Carbapenemases

Fifty-one (93%) and 50 (90%) *K. pneumoniae* isolates were labeled as CPKP, according to the results of the mCIM and Carba NP tests, respectively. However, 48 (84%) isolates were identified as CPKP with the MHT.

Molecular Analysis of Antimicrobial Resistance Genes in the *K. pneumoniae* Isolates

K. pneumoniae comprised the vast majority of the carbapenem-resistant isolates. Accordingly, they were subjected to further analyses to determine their genetic background. PCR screening revealed that 30, 12, 2, and 1 isolates were harboring carbapenemase genes (*bla*_{OXA-48}, *bla*_{NDM}, *bla*_{IMP}, and *bla*_{VIM}), respectively. Furthermore, β -lactamases genes (*bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M}) were detected in 28, 16 and 24 *K. pneumoniae* isolates, respectively. Meanwhile, 63% of the *K. pneumoniae* isolates co-harbored more than one carbapenemase gene. It is worth mentioning that among the *K. pneumoniae* isolates, an isolate (no.134) co-harbored (*bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP-1}). This specific strain was isolated from a blood specimen of a patient with hematologic malignancy admitted to the ICU. Furthermore, it was

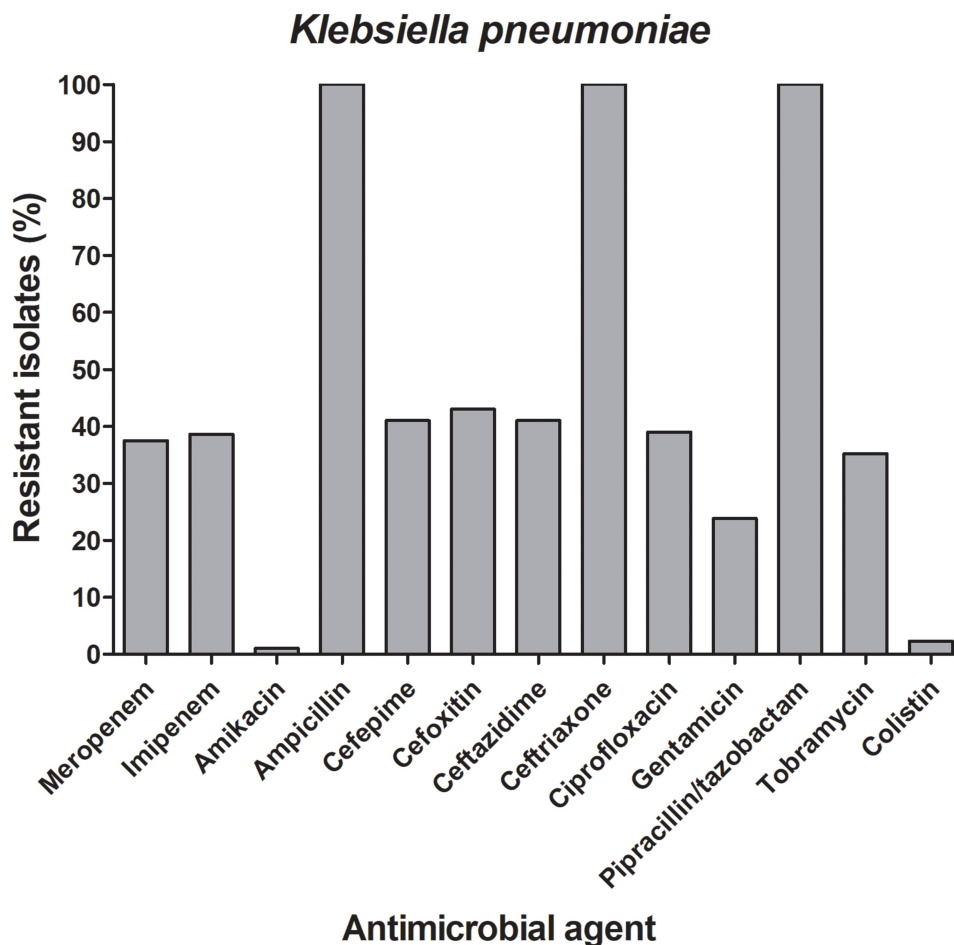


Figure 1 Frequencies of the antimicrobial resistance among the carbapenem-resistant *K. pneumoniae* isolates. A bar graph presenting the percentages of the resistance to 14 different antibiotics among the 52 carbapenem-resistant *K. pneumoniae*. The chart was generated using GraphPad Prism v5.

characterized by being MDR carbapenemase-producer isolate that was phenotypically positive by all the MHT, CarbaNP, and mCIM tests. It also co-harbored the ESBLs genes (*bla_{SHV-1}* and *bla_{CTX-M15}*) and showed MIC value of 16 and 32 µg/mL to meropenem and imipenem, respectively. To the best of our knowledge, this is the first report of this gene combination in a strain isolated in Egypt. The distribution of the different analyzed resistance genes in all the *K. pneumoniae* isolates among gender, different clinical specimens, and wards is presented in Table 2. To provide a better representation of the frequencies of the carbapenemase and ESBLs genes among the carbapenem-resistant *K. pneumoniae* isolates the data is illustrated in Figure 2. The most common gene combinations were (*bla_{OXA-48}*, *bla_{TEM}*, *bla_{SHV}*, and *bla_{CTX-M}*) and (*bla_{OXA-48}*, *bla_{TEM}*). This was followed by the (*bla_{OXA-48}*, *bla_{TEM}*, and *bla_{CTX-M}*) combination. On the other hand, the most common single gene, not present in a combination with other carbapenemase and ESBLs genes, was the *bla_{NDM}* followed by *bla_{OXA-48}* and *bla_{TEM}*.

Nucleotide sequences analyses of some of the obtained PCR products were compared with the non-redundant genome sequences available in the GenBank database.

These analyses revealed the prevalence of *bla_{OXA-48}*, *bla_{NDM-1}*, *bla_{IMP-1}*, *bla_{VIM-1}*, *bla_{TEM-1}*, *bla_{SHV-1}*, and *bla_{CTX-15}* among the studied isolates. Overall, analysis of the distribution of the genetic resistance determinants indicated that the *bla_{OXA-48}* is widely spread among the included carbapenem-resistant *K. pneumoniae* isolates (30/52; 58%). Therefore, these strains were further investigated molecularly for their clonal relatedness.

ERIC-PCR Analysis

Dendrogram analysis of the ERIC-PCR genotyping revealed three clusters of the *bla_{OXA-48}*-harbouring *K. pneumoniae*, one major cluster (I) included 27 isolates and the other 3 isolates demonstrated unique patterns distributed among the other 2 clusters (II and III) (Figure 3). Sixty percent of cluster I isolates exhibited similarity $\geq 95\%$; however, the 3 other isolates were $\leq 85\%$ similar (Figure 3). The discriminatory power for the ERIC analysis was 0.2.

PFGE Analysis

The PFGE analysis of the 30 *bla_{OXA-48}*-harbouring *K. pneumoniae* isolates revealed 28 distinct PFGE profiles (pulsotypes) as illustrated in the dendrogram (Figure 4). The

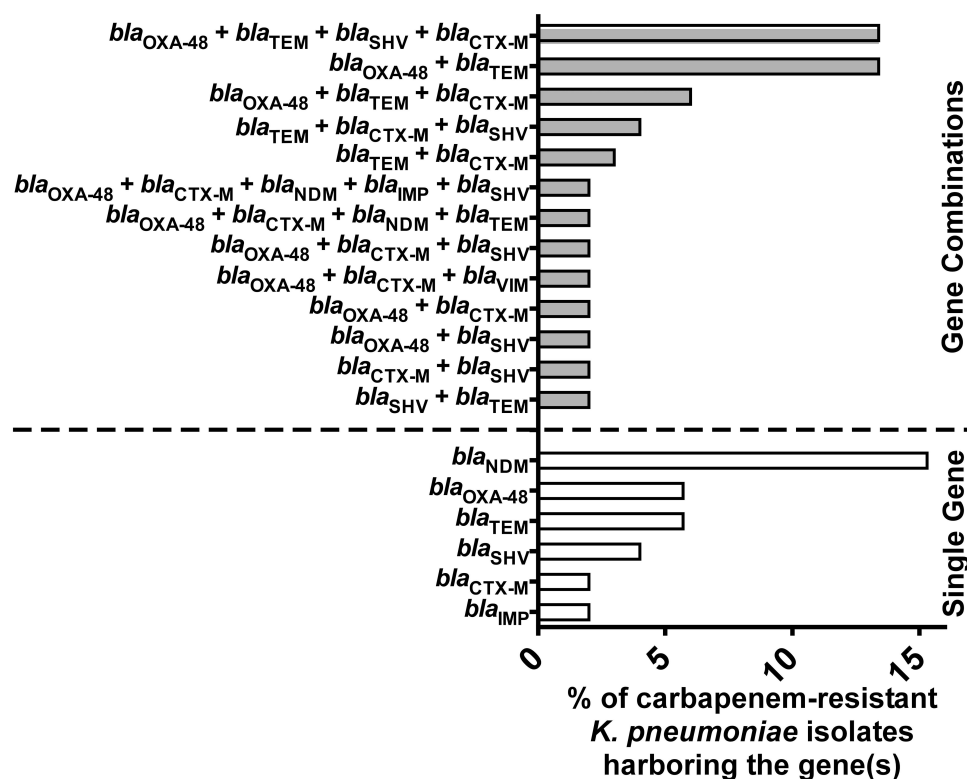


Figure 2 Distribution of carbapenemases and ESBLs encoding genes among the carbapenem-resistant *K. pneumoniae* isolates. A bar graph showing the percentages of carbapenem-resistant *K. pneumoniae* isolates (n=52) harboring genes encoding carbapenemases (*bla_{OXA-48}*, *bla_{NDM}*, *bla_{VIM}*, and *bla_{IMP}*) and ESBLs (*bla_{CTX-M}*, *bla_{TEM}*, and *bla_{SHV}*) either in combination (grey bars) or as single genes (white bars).

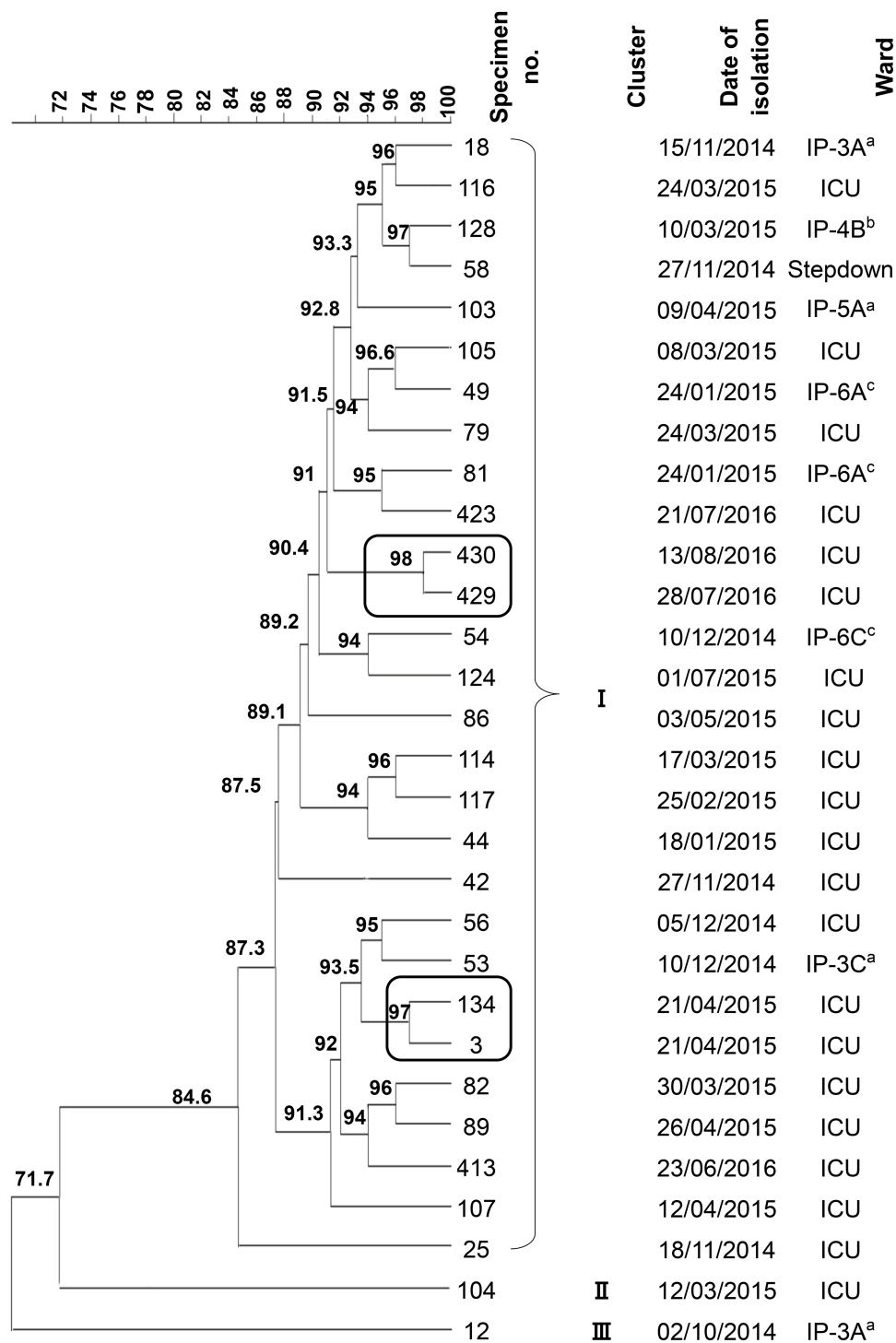


Figure 3 ERIC-PCR profiles of carbapenem-resistant *K. pneumoniae* harboring the *bla*_{OXA-48} gene. A dendrogram was generated from the ERIC-PCR typing of 30 *K. pneumoniae* clinical isolates. The analysis was performed using BioNumerics fingerprint data software v7.62 and the unweighted pair group method with arithmetic means (UPGMA) clustering method and the dice similarity coefficient with 1% optimization and 1.5% position tolerance. The different clusters are designated by Roman numerals (I–III). The numbers written on the branches indicate the similarity percentage of the different isolates. On the right side of the dendrogram detailed results including the number of each isolate, clusters, date of isolation, and hospital wards. The alphabets indicate the type of the hospital wards; a: hematology ward, b: solid tumor ward, c: surgery ward.

Abbreviations: IP, in-patient; ICU, intensive care unit; stepdown unit, intermediate care unit.

P17 was the most common pulsotype (3/30, 10% of isolates), ie, isolates 86, 429, and 430 showed an indistinguishable pattern. Whereas, another 2 strains exhibited 99%

similarity coefficient (3 and 134) that were suggested to be clonally related to the former 3 isolates (86, 429, and 43). The PFGE dendrogram identified 5 clusters (A–E) that

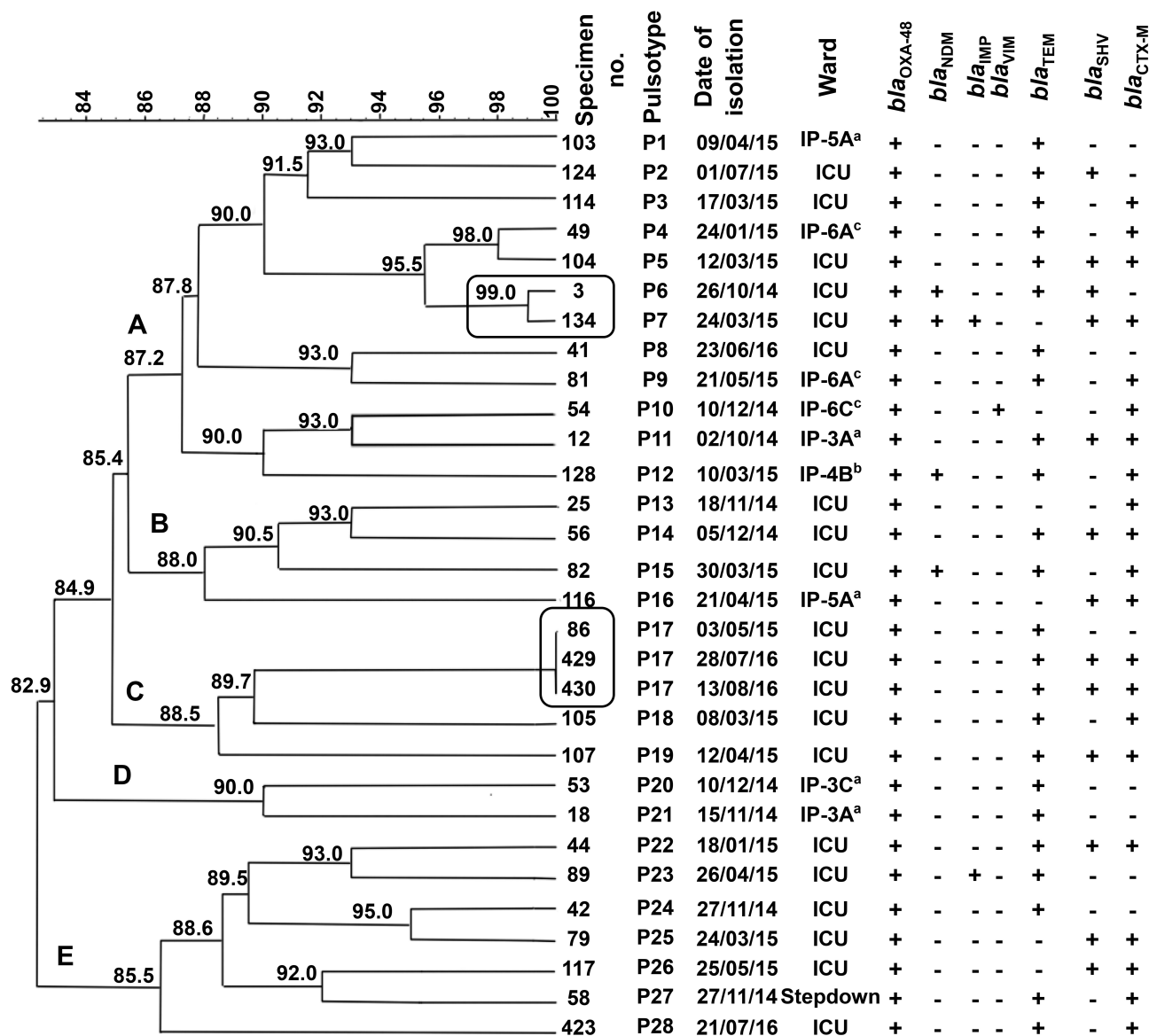


Figure 4 PFGE typing of the *bla*_{OXA-48}-harbouring carbapenem-resistant *K. pneumoniae* isolates. A dendrogram was generated from the PFGE patterns of XbaI digested chromosomal DNA of 30 carbapenem-resistant *K. pneumoniae* clinical isolates. The comparison was performed with the BioNumerics fingerprint data software v7.62 using the UPGMA clustering method and the dice similarity coefficient with 1% optimization and 1.5% position tolerance. PFGE pulsotypes (P) were assigned to isolates according to Tenover criteria.²⁷ The numbers written on the branches indicate the similarity percentage of the different pulsotypes. The black frame indicates the isolates that were indistinguishable and assumed to be the same clone. The different clusters are designated by letters (A–E). On the right side of the dendrogram detailed results for PFGE including the code of each isolate, pulsotypes, date of isolation, hospital wards, and screened carbapenemases and ESBLs determinants (*bla*_{OXA-48}, *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{CTX-M}, *bla*_{TEM}, and *bla*_{SHV}). The (+) and (-) signs indicate present and absent, respectively. The alphabets indicate the type of the hospital wards; a: hematology ward, b: solid tumor ward, c: surgery ward.

Abbreviations: IP, in-patient; ICU, intensive care unit; stepdown unit, intermediate care unit.

exhibited $\geq 85\%$ similarity coefficient. Cluster A was predominating, as it included 12 isolates, clusters B, C, D, and E included 4, 5, 2, and 7 isolates, respectively. However, 9/30 (30%) of the isolates demonstrated a similarity coefficient $\geq 95\%$. The discriminatory power for this analysis was 0.76.

The Conjugation Experiments

The *bla*_{OXA-48} gene from the two donor strains was successfully transferred to the recipient *E. coli* K12.

PCR results indicated that the gene was successfully transferred in both conjugations. The meropenem MIC values determined were consistent with the transfer of carbapenemase expression. As the MIC value of the recipient strains changed from ≤ 0.5 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ with the donor strains 128 and 116, respectively. Accordingly, this experiment revealed that the plasmids harboring the *bla*_{OXA-48} gene in some of the investigated isolates are transferrable via conjugation

and capable of conferring resistance to meropenem to *E. coli* K12.

Discussion

In 2017, the World Health Organization (WHO) defined CRE as one member of the “critical priority list of antibiotic-resistant pathogens”.³³ In 2018, the European Centre for Disease Prevention and Control (ECDC) conducted a review to update the risk assessment of CRE, as it represents an important threat to patients in healthcare systems.³⁴ A recent study in Egypt reported that there is a continuous increase in the prevalence of CRE in the country, with at least one CRE isolate in 64% from 72 hospitals during the study period.³⁵ As reported in other previous studies in Egypt, our study also concluded that the predominant species among the isolated CRE was MDR *K. pneumoniae*, which represents a frightening causative agent of different types of infections among hospitalized pediatric cancer patients.^{35–38} It was reported that class D carbapenemases (bla_{OXA-48} = 58%) are predominant in *K. pneumoniae* followed by the bla_{NDM-1} gene, in several studies conducted in different countries notably in the Mediterranean region including Spain, France, Turkey, Tunisia and Egypt.^{20,21} Furthermore, the majority (41.8%) of the *K. pneumoniae* isolates in the current study harbored one or more carbapenem resistance determinants (Figure 2) that agreed with previously reported studies.^{40–42} The unusual combination of the 3 carbapenemase genes (bla_{OXA-48} , bla_{NDM-1} , and bla_{IMP-1}), was among our alarming findings, especially it was not reported before among isolates in Egypt. Interestingly, several studies from China, Japan, and multiple countries in the Mediterranean region including; France, Morocco, Egypt, Algeria, Tunisia, and Spain reported the presence of single bla_{IMP-1} or in combination with either bla_{NDM-1} or bla_{OXA-48} carbapenemase genes.^{24,25} However, the current triple gene-combination has not been previously reported. Ten percent of the CRKP isolates were non-carbapenemase producers, which could be explained by the overexpression of AmpC or ESBLs combined with permeability alteration (deficient expression or loss of porins) and/or other carbapenemases that were not identified.⁴³

Regarding, the CarbaNP and mCIM phenotypic tests results, both tests indicated that the numbers of positive isolates are higher than that detected by the MHT especially for those isolates encoding for the bla_{OXA-48} . Hence, our findings are consistent with other previously reported

results regarding the rates of detecting positive results by these three assays.^{47,48}

The present study revealed that 49% of the recovered isolates were MDR. However, amikacin, colistin, and tigecycline were still effective against most of the *K. pneumoniae* isolates (Figure 1), similar to other studies conducted in Egypt and elsewhere.⁴⁶ Although there is a marked increase in the resistance of amikacin, colistin, and tigecycline in recent studies in Egypt, they are still effective against carbapenem-resistant Enterobacteriaceae.^{37,39,47} Molecular typing is a useful tool for determining the genetic relatedness of bacteria. This is particularly important in the investigation of the epidemiology of several bacterial infectious diseases accompanied by HAI.⁴⁸ To understand how bla_{OXA-48} is disseminated through the study hospital, two different DNA-based fingerprinting methods were used to compare the isolates at the molecular level. The dendrogram relationships of the 30 isolates for both the ERIC and PFGE analyses revealed high genetic diversity among the investigated isolates except for two pairs of isolates that were closely related. Since the 30 genotyped isolates co-harbored a diverse range of resistance determinants, therefore they exhibited resistance to carbapenems with different mechanisms that are considered an alarm for the potential dissemination of these clones among the hospital wards. According to the aforementioned results, when compared with PFGE, ERIC-PCR displayed a much lower discriminatory power. The isolates were categorized by the PFGE among a larger number of clusters compared to ERIC-PCR typing. A dominant PFGE pattern with $\geq 90\%$ similarity coefficient was observed in 26 isolates, while the other isolates displayed unique PFGE patterns ranging from 85% to 90% similarity. These findings indicate the emergence and spread of these resistant isolates across the hospital wards. Moreover, they also indicate that cross-transmission is not restricted to a certain ward or time. For example, the 3 isolates (86, 429, and 430) showed the same pulsotype with 100% similarity and were isolated from the same ward but almost one year apart regarding the date of sample collection. Additionally, the latter isolates revealed the same resistance determinants (bla_{OXA-48} , bla_{TEM-1} , bla_{SHV-1} , and $bla_{CTX-M15}$) combination except for (bla_{SHV-1} and $bla_{CTX-M15}$) in isolate no. 86. Another example was isolate no.134 that exhibited 99% similarity and co-harbored 5 different resistance determinants bla_{OXA-48} , bla_{NDM-1} , bla_{IMP-1} , bla_{SHV-1} , and $bla_{CTX-M15}$ (Figure 4).

The two molecular typing methods revealed that 5 *K. pneumoniae* isolates (429, 430, and 86) and (3 and 134) were categorized as a unique subtype. Also, they

exhibited MIC values between 4 µg/mL and ≥ 16 µg/mL for meropenem and imipenem, respectively. Interestingly, we observed that the aforementioned 5 isolates (86, 429, 430, 3, and 134) were obtained from the hospital ICUs. Similar to our study, recent two studies were conducted also in Egypt that reported the prevalence of diverse clones from CRKP using the PFGE and BOX PCR as molecular typing methods, respectively.^{37,49}

It could be inferred from using the two different typing methods that ERIC-PCR method might be less discriminatory in the evaluation of distantly related isolates. Conversely, it could be a useful tool for evaluating isolates that are closely related genetically.

Since 57% of the total *K. pneumoniae* isolates were recovered from the ICU, therefore, the increase in the ICU admission in our study could highlight the increase of bacterial infection with *K. pneumoniae*, as it can form a biofilm. Thus, allowing its adherence to different invasive procedures that are often used in that ward.⁵⁰ Those results are consistent with other studies conducted in Egypt, Colombia, the United States, and Greece, among pediatric patients, admitted to the ICU.^{16,37,51} On the other hand, a study in the Netherlands reported that admission and long ICU stay, use of invasive medical devices and carbapenem as an empirical therapy were the most significant risk factors for CRE acquisition by hospitalized patients particularly vulnerable patients.⁵² Accordingly, infection control measures should focus on the ICU, where the relevant patients at risk for either colonization and/or infection with HAI causing microorganisms are common.

The 30 genotyped CPKP isolates were ESBLs producers and showed significant resistance to ampicillin, cephalosporins, fluoroquinolones, and carbapenems, but exhibited differential susceptibility to amikacin (1%) and colistin (2.3%). These findings highlighted the risk of clonal dissemination of *bla*_{OXA-48}-harboring *K. pneumoniae* among the hospital wards similar to a study conducted in the United Arab Emirates, which reported comparable results.⁵³ However, in Japan, Khalifa and co-workers reported no clonal relationship among carbapenemase-producing isolates.⁴⁰ Furthermore, a study in Italy reported that there is a relationship between the hospital environment, and the increased prevalence of resistant bacteria causing infections often encountered in the ICU setting, followed by non-ICU hospital wards and outpatient hospital settings.⁵⁴ In 2016, a study in France reported the persistence of OXA-48-

producing *K. pneumoniae* for 20 months in a hospital ICU environment.⁵⁵

The dissemination of some clonally-related isolates among the hospital's different wards during the study period is an indication of the probability of cross-transmission of these resistant isolates in the hospital. The spread of the carbapenemases is linked mainly to mobile genetic elements such as plasmids, transposons, and integrons that carry not only resistance but might also harbor virulence determinants. Thus, facilitating transmission of resistant determinants between the same and different bacterial species including the bacteria present in the human microbiome.⁵⁶ Consequently, this could affect the bacterial ability for colonization and infection within the host.⁵⁷ The *bla*_{OXA-48} like genes are always carried on a broad range of plasmids such as IncH, IncA/C, IncX3, and ColKP3.⁵⁸ The genes encoding for these resistance mechanisms are transferable between bacteria. Hence, there is a high risk of dissemination and potential healthcare-associated outbreaks of Enterobacteriaceae with these resistance mechanisms.⁵⁷ In the current study, the conjugation experiment for the *bla*_{OXA-48} harboring plasmids from representative isolates was successful consistent with other studies.⁵⁹⁻⁶¹

Based on our molecular typing results, both methods can be used for the determination of the clonal relationships of *K. pneumoniae* strains. Although ERIC-PCR is a more rapid and easy method compared to PFGE, its discriminatory power seemed to be less than that of PFGE.

The current study presents the molecular epidemiological situation of the MDR CRKP spread among hospitalized pediatric cancer patients in a major hospital in Egypt. It is notable from our findings that there might be dissemination of clonally-related strains among the ICU wards in the hospital. Therefore, the implementation of strict control measures for CRKP among the healthcare wards requires reliable and rapid identification methods. Additionally, hospital settings should have a protocol for effective screening for CPE, especially in high-risk patients. Further studies are required to investigate the sequence type of the disseminating clones in the hospital settings and consider the carbapenemases' impact on the fitness and virulence in Enterobacteriaceae.

Ethics Approval

The study approval was obtained from the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (approval no. MI-1203).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work".

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