Microbiology Research Journal International

24(3): 1-16, 2018; Article no.MRJI.42911
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Carbapenemase-Encoding Genes in *Pseudomonas aeruginosa*: Multiplex PCR Detection, Microarray Based Detection and Gene Sequencing

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Authors' contributions

This work was carried out in collaboration between all authors. Author AKEE designed the study, wrote the protocol, performed the results' analysis, managed the literature searches and wrote the first draft of the manuscript. Author HMAS reviewed the protocol and reviewed study results. Author BMEB collected samples, performed the practical investigations and literature search. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2018/42911
Editor(s):
(1) Xing Li, Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, Mayo Clinic College of Medicine, USA.
Reviewers:
(1) Gokben Ozbey, Firat University, Turkey.
(2) P. Hema Prakash, GITAM Institute of Medical Sciences & Research, GITAM University, India.
(3) George Masifa, Mbale Clinical Research Institute, Uganda.
Complete Peer review History: http://www.sciencedomain.org/review-history/25679

Original Research Article

Received 12th May 2018
Accepted 20th July 2018
Published 25th July 2018

ABSTRACT

Background: In 2017 the World Health Organization (WHO) classified carbapenem-resistant *Pseudomonas* among the most critical multidrug-resistant bacteria for urgent attention.
Aims: Evaluation of two molecular methods for detection of carbapenemase encoding genes in carbapenem resistant *Pseudomonas aeruginosa* (CRPA), and alignment of detected gene sequences with gene bank data.
Materials and Methods: Twenty two previously detected CRPA isolates were tested for bacterial identification and antibiotic sensitivity by verigene-nanosphere technique. Verigene-nanosphere microarray based assay and multiplex PCR were tested for detection of carbapenem encoding genes. Carbapenemase encoding genes sequencing of parent strain and its UV mutant followed by nucleotide sequence alignments were conducted. Bacterial resistance to carbapenem was tested after induced plasmid curing by sodium dodecyl sulfate (SDS) and heat.

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Results: Typical results of conventional PCR were obtained using multiplex PCR for a set of genes, including IMP, VIM, SME. Verigene-nanosphere detected 3, 0, 0 among 5, 4, 11 known positive strains for VIM, IMP, KPC genes respectively. Carbapenem resistance was preserved after treating CRPA strain by heat or SDS. The nucleotide sequence alignments of VIM, IMP and KPC genes showed relatedness with many Gram negative species. VIM gene was lost in UV mutant and the IMP and KPC genes were preserved, but 1-2% sequence modification occurred without a change in resistance to imipenem and meropenem.

Conclusion: The developed multiplex PCR detected successfully a set of carbapenem encoding genes, while microarray based verigene-nanosphere failed to detect most of the genes under the current experimental conditions. The nucleotide alignments of VIM, IMP and KPC genes revealed that these gene sequences are distributed among species of Gram negative bacteria. IMP and KPC genes were preserved in UV mutant with no damage, repaired damage or little modification of the nucleotide sequence and meanwhile carbapenem resistance was preserved.

Keywords: Carbapenem resistance; Pseudomonas aeruginosa; carbapenemase encoding genes; microarray; multiplex PCR.

1. INTRODUCTION

The use of carbapenems is the last resort for treating infections caused by many bacteria, especially *Pseudomonas aeruginosa*, which has intrinsic resistance to a large variety of antimicrobials and can acquire resistance determinants by horizontal transfer of mobile genetic elements from other bacteria [1-3]. However, the threat of multidrug-resistant microorganisms, including those resistant to carbapenems, becomes an international public health crisis [4-7].

Carbapenem resistance is due to multiple mechanisms, including production of carbapenemases, over-expression of the efflux pump, or loss of outer membrane porins plus production of extended spectrum ß-lactamase or AmpC ß-lactamase [8]. Among these resistance mechanisms, production of carbapenemase is the most important because it was associated with higher mortality rates compared with noncarbapenemase-producing CRPA [9]. The reported carbapenemases in *P. aeruginosa* included non metallo-enzyme carbapenemase (NMC), *Serratia marcescens* enzyme (SME), *Klebsiella pneumoniae* carbapenemase (KPC), imipenem hydrolyzing ß-lactamase (IMI), Guiana extended-spectrum ß-lactamase (GES), imipenemase (IMP), Verona integronen coded metallo-ß-lactamase (VIM), German imipenemase (GIM), Sao Paulo metallo-ß-lactamase (SPM), New Delhi metallo- ß -lactamase (NDM), and oxacillinase-48 (OXA-48), among which VIM has been the most predominant [10].

The authors previously detected KPC, VIM, IMP genes in CRPA isolates, while SME, NDM-1(using two pairs of primers) and OXA genes were not detected [11]. In the current study two molecular methods, namely multiplex PCR and microarray based "verigene nanosphere" techniques were compared to conventional PCR for detection of carbapenemase encoding genes. PCR products of carbapenemase encoding genes were subjected to gene sequencing and aligned with gene bank sequences. Selected CRPA strains were induced for gene mutation by UV radiation and the resultant mutants were tested for preservation of carbapenemase encoding genes and their gene sequence stability. On the other hand the effect of SDS and thermal treatment on CRPA strains was also investigated regarding preservation of carbapenem resistance and associated encoding genes.

2. MATERIALS AND METHODS

2.1 Samples and Bacterial Isolates

Clinical samples (Urine, pus, blood, sputum, pleural effusion, etc) were randomly collected from different hospitals in Kuwait. The fresh collected samples were inoculated on the suitable media and the pure isolates were kept in brain Heart Infusion broth medium (Difco laboratories, England) with 15% glycerol (Sigma, USA) at -70°C.

2.2 Bacterial Identification

Morphological characteristics were analyzed according to the Bergey's Manual of Systematic Bacteriology [12]. Standard manual physiological and biochemical identification and VITEK-2

automated identification (BioMérieux, USA) were carried out for detection of *Pseudomonas aeruginosa*.

2.3 Antibiotic Sensitivity and MIC Determination

Antimicrobial disc diffusion susceptibility test on Mueller Hinton Agar (Oxoid, England) was performed by Kirby-Bauer method [13-15]. Minimum Inhibitory Concentration (MIC) was determined basically by micro-dilution method (standard antibiotics from GlaxoSmithKline, Egypt) according to previous publications [15-17]. MIC was also determined by VITEK-2 (automated instrument). E-TEST was also used for MIC determination of meropenem and imipenem.

After exposure to UV radiation, selected mutant strains of *Pseudomonas aeruginosa* were tested again for antibiotic sensitivity, MIC (as shown below).

2.4 DNA Extraction and PCR Amplification

Depending on the previous identification and antibiotic sensitivity testing that were carried out on clinical bacterial isolates, eighty CRPA strains were identified. Twenty two CRPA strains were randomly selected and subjected to DNA extraction for further investigations. DNA was extracted using the QIAamp DNA mini kit according to its manufacturer's instructions. The PCR master mix was prepared according to Emerald Amp GT PCR master mix kit (Takara, Japan, code no. RR310A). Class A, class B and class D carbapenemase genes were amplified using the primers and conditions previously described [18]. The primers (sigma, USA) were used with an adaptation of the instructions provide with Emerald Amp GT PCR master mix (Takara, Japan) (Table 1).

2.5 Multiplex PCR Amplification

DNA was extracted using the QIAamp DNA mini kit according to its manufacturer's instructions. The PCR master mix was prepared according to Emerald Amp GT PCR master mix kit (Takara, Japan, code no. RR310A). Depending on the PCR product sizes, selected carbapenemase encoding genes were tested for detection by single reaction multiplex PCR with an adaptation of previously described conditions [18-24]. The primers (sigma, USA) were used with an adaptation of the instructions provide with Emerald Amp GT PCR master mix (Takara, Japan) (Table 2).

2.6 Induction of Bacterial Mutation by UV Radiation

According to the PCR results, selected isolates were exposed to UV radiation (Ultraviolet transilluminator, Cole Parmer, USA) for enhancement of DNA mutations. Fifty ml of overnight culture was harvested and re-suspended in 10 ml broth. A half ml of the broth was transferred to each of 4 plates and 2 ml saline was added. One plate was UV control, 3 plates were exposed to UV radiation (450 nm, 20 cm distance from the source) for 3, 5, 7 minutes respectively. Serial dilutions were performed from bacterial cells exposed to different times of UV. Inoculations from dilutions of 10^{-6}, 10^{-7}, 10^{-8} from UV control, 10^{-4}, 10^{-5}, 10^{-6} from 3 minutes exposure, 10^{-2}, 10^{-3}, 10^{-4} from 5 minutes exposure and 10^{-1}, 10^{-2} from 7 minutes exposure. Inoculations were incubated anaerobically from at least 10 colonies of each dilution and selected for further testing to obtain the best mutants.

2.7 Induction of Bacterial Plasmid Curing by Treatment with Sodium Dodecyl Sulfate and Heat

Selected CRPA strains (Parent clinical strains 52, 57) were treated by sodium dodecyl sulfate (SDS "Sigma, USA") and heat respectively to enhance plasmid curing and testing selections the resultant colonies for variation in carbapenem resistance.

Heat treatment was performed by incubating different dilutions of bacterial suspensions in brain heart infusion broth at 42°C for 48 hours, and then random colonies from different dilutions were picked and subjected to further experiments.

On the other hand, 1 ml of brain heart infusion (BHI) broth containing 10% SDS was inoculated with 10 *µl* of an overnight culture. Incubation overnight was carried out at 37C. Dilutions were made then plated on the solid medium and incubated overnight to obtain isolated colonies. The colonies were inoculated separately on slants and incubated overnight. The separate colonies (mutants) were subjected to further analysis.

Table 1. Polymerase chain reaction primers and conditions used for detection of carbapenemase encoding genes

Class	Target gene	Sequence (5'-3')	Amplicon size (bp)	Reference no.	PCR conditions					
					Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Class A	*SME*	AACGGCTTCATTTTTGTTTAG GCTTCCGCAATAGTTTTATCA	820bp	19	94°C5min.	94°C30 sec.	50°C45sec.	72°C45 sec.	35	72°C10 min.
	KPC	ATGTCACTGTATCGCCGTCT TTTTCAGAGCCTTACTGCCC	892bp	20	94°C5 min.	94°C30 sec.	55°C45 sec	72°C45 sec	35	72°C10 min.
	IMP	CATGGTTTGGTGGTTCTTGT ATAATTTGGCGGACTTTGGC	488bp	21	94°C5 min.	94°C30 sec.	50°C45 sec	72°C45 sec	35	72°C10 min.
Class B	*VIM*	AGTGGTGAGTATCCGACA ATGAAAGTGCGTGGAGAC	280bp	22	94°C5 min.	94°C15 sec.	50°C30 sec.	72°C30 sec.	35	72°C7 min.
	NDM-1	GGCGGAATGGCTCATCACGA CGCAACACAGCCTGACTTTC	287bp	23	94°C5 min.	94°C15 sec.	55°C30sec.	72°C30 sec.	35	72°C7 min.
Class D	*OXA*	CAGCACACTTCCTATCTC CCGCAACCATCCCCTCTT	293bp	23	94°C5 min.	94°C15 sec.	50°C30 sec	72°C30 sec	35	72°C7 min.
		TTTTCTGTTGTTTGGGTTTT TTTCTTGGCTTTTATGCTTG	519bp	24	94°C5 min.	94°C30 sec.	48°C45 sec	72°C45 sec	35	72°C10 min.

Table 2. Multiplex Polymerase chain reaction primers and conditions used for detection of selected carbapenemase encoding genes

Class	Target gene	Sequence (5'-3')	Amplicon size (bp)	Reference no.	PCR Conditions					
					Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Class A	*SME*	AACGGCTTCATTTTTGTTTAG GCTTCCGCAATAGTTTTATCA	820 bp	19	94°C 5min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	35	72°C 10min.
Class B	*IMP*	CATGGTTTGGTGGTTCTTGT ATAATTTGGCGGACTTTGGC	488 bp	21	94°C 5 min.	94°C 30 sec.	50°C 45 sec	72°C 45 sec	35	72°C 10min.
	VIM	AGTGGTGAGTATCCGACA ATGAAAGTGCGTGGAGAC	280 bp	22	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 30 sec.	35	72°C 10min.

2.8 Antibiotic Sensitivity, MIC and PCR Testing for Bacterial Mutants

After exposure to UV radiation, selected mutant strains of *Pseudomonas aeruginosa* were tested for antibiotic sensitivity, MIC and PCR detection of carbepenemase genes as described above.

2.9 Microarray Based Verigene BC-GN Nanosphere for Bacterial Identification and Detection of Carbapenemase Genes

Twelve CRPA strains harboring carbapenemase encoding genes (according to conventional PCR results) were subjected to bacterial identification and detection resistance encoding genes using verigene-nanosphere technique as described by the manufacturer (Nanosphere inc., USA) [25].

2.10 Sequencing of the PCR Products

PCR products of selected clinical strains of CRPA (Parent strain 52 isolated by the authors from clinical samples in Kuwaiti hospitals and its UV mutant strain 42) were subjected for sequencing. These strains selected to cover different detected carbapenemase genes and to test any effect on the gene sequence after the inducted mutation.

The dideoxyribonucleoside chain termination procedure was employed for sequencing the double-stranded DNA [26].

The capillary array was filled with POP-7™ polymer, a medium that separates the DNA fragments. The procedure was followed according to Applied Biosystems 3730/3730xl DNA Analyzers User Guide (PN 4331468, Applied Biosystems, USA).

The fluorescently labeled DNA was loaded into the capillary array by a short period of electrophoresis called electrokinetic injection. The capillary array was rinsed with water to remove any sample adhering to the sides.

The autosampler brought the buffer reservoir to the capillary array for electrophoresis (48-capillary array for the 3730 DNA Analyzer, or 48- and 96-capillary arrays for the 3730xl DNA Analyzer).

The labeled DNA sequence fragments were separated by size as they travel through the polymer-filled capillary array (electrophoresis). As they reach the detection window, the laser beam excites the dye molecules and causes them to fluorescence (electrophoresis).

The fluorescence emissions from 48 or 96 samples were collected simultaneously and spectrally separated by a spectrograph. The fluorescence emissions were focused as columns of light onto the CCD camera.

The 3730/3730xl Data Collection software reads and interprets the fluorescence data, then displays the data as an electropherogram.

2.11 Nucleotide Sequence Alignments

The BLAST program was used that compares a nucleotide query sequence to a database of nucleotide sequences at the NCBI.

3. RESULTS AND DISCUSSION

3.1 Results

Depending on the PCR product sizes VIM, IMP and SME genes were selected to be tested by multiplex PCR in selected isolates that were positive for VIM, IMP genes by conventional PCR method. The results of multiplex PCR were typical to that of conventional PCR. In other words, there was 100% agreement between conventional PCR and multiplex PCR results for detection of VIM, IMP and SME (Fig. 1).

The clinical strains were isolated from different hospitals and identified. *P. aeruginosa* strains were tested for sensitivity to meropenem and imipenem antibiotics. Based on CRPA selection criteria, 80 CRPA strains were obtained and stored for further studies. 22 CRPA strains were tested by conventional PCR, 12 of them were positive to at least one of the carbapenemase encoding genes. These 12 strains were also tested for detection of carbapenemase encoding genes by verigene-nanosphere technique. Among 12 CRPA isolates harbor one or more of carbapenemase encoding genes that were detected by conventional PCR method, 3 of them were positive for VIM gene only using microarray based assay (Verigene BC-GN nanosphere). Actually, the three isolates were positive for VIM gene among known 5 VIM harboring strains that were detected by PCR. No IMP or KPC genes were detected by verigene-nanosphere among 11 strains and 4 strains that are harboring KPC

and IMP respectively. In other words, in comparison to the conventional PCR, the detection of VIM gene by Verigene BC-GN nanosphere was 3/5 (66.6%), the detection of KPC by Verigene BC-GN nanosphere was 0/11 (0%) and the detection of IMP by Verigene BC-GN nanosphere was 0/4 (0%), (Table 3). On the other hand all of the 22 CRA isolates were identified by conventional methods and Verigene BC-GN nanosphere method as *P. aeruginosa* and resistant to both imipenem and meropenem.

For plasmid curing experiments using heat or SDS treatment, the experiment was designed to detect genes by verigene-nanosphere before and after treatment. There were no observed effect on resistance pattern or detected carbapenemase genes. Two selected CRPA isolates (harbored KPC, IMP and VIM genes as detected by PCR, harboring only VIM as detected by verigene-nanosphre) were treated with sodium dodecyle sulfate (SDS) preserved the resistant to both imipenem and meropenem associated preservation of VIM gene according to verigene-nanosphere results. The same two CRPA isolates were treated also with heat, then showed resistance to imipenem and meropenem associated with VIM gene preservation (Table. 4), however plasmid profiling are not performed in all cases.

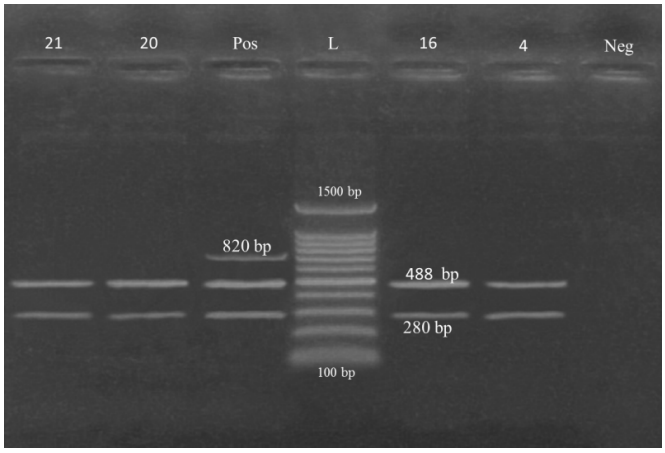

Fig. 1. Agarose gel electrophoresis showing Multiplex PCR product bands of carbapenemase encoding genes for selected genes (IMP {PCR product: 488 bp}, VIM {PCR product: 280 bp}, SME {PCR product: 820 bp}) in representative samples

Table 3. Comparison between detected genes by PCR technique and Microarray based assay (Verigene BC-GN Nanosphere)

Parent strain code	Detected genes by conventional PCR technique	Detected genes by Verigene BC-GN Nanosphere
8	KPC	No detected genes
13	KPC	No detected genes
22	KPC,IMP,VIM	No detected genes
25	VIM	No detected genes
41	KPC	No detected genes
43	KPC	No detected genes
44	KPC	No detected genes
51	KPC	No detected genes
52	KPC,IMP,VIM	VIM
55	KPC	No detected genes
56	KPC,IMP,VIM	VIM
57	KPC,IMP,VIM	VIM

Gene detection using Verigene BC-GN nanosphere was 66.6%, 0% and 0% for each of VIM, KPC and IMP genes respectively, in comparison to the conventional PCR

Table 4. Effect of sodium dodecyle sulfate or heat treatment on selected carbapenem resistant *P. aeruginosa* strains

Parent isolate number	Antibiotic Sensitivity			PCR Detected genes	Verigen-Nanosphere Detected genes before SDS or heat treatment	Antibiotic Sensitivity after SDS or heat treatment			Verigene-Nanosphere Detected genes after SDS or heat treatment
	Antibiotic	MIC	Interpretation			Antibiotic	MIC	Interpretation	
52	Imipenem	>=16	R	KPC, IMP,	VIM	Imipenem	>=16	R	VIM
	Meropenem	>=16	R	VIM		Meropenem	>=16	R	
57	Imipenem	>=16	R	KPC, IMP,	VIM	Imipenem	>=16	R	VIM
	Meropenem	>=16	R	VIM		Meropenem	>=16	R	

Table 5. Best gene sequence alignments of Carbapenemase encoding genes

Sequenced PCR gene product (Parent Strain 52)	Alignments description	Identity	Gap	Strand	Gene bank accession no.
VIM (Reverse primer)	*Pseudomonas aeruginosa* strain Ps-1 metallo-beta-lactamase VIM-11 gene, partial cds sequence	241/252(96%)	4/252(1%)	Plus/Minus	GQ221779.1
VIM (Forward Primer)	*Klebsiella pneumoniae* strain NRZ-28192 subclass B1 metallo-beta-lactamase VIM-46 (blaVIM) gene, blaVIM-46 allele, complete cds	227/231(98%)	2/231(0%)	Plus/Plus	KX811538.1
KPC (Reverse Primer)	Pseudomonas aeruginosa strain S04 90 genome	555/557(99%)	1/557(0%)	Plus/Plus	CP011369.1
KPC (Forward Primer)	Pseudomonas aeruginosa strain S04 90 genome	555/559(99%)	2/559(0%)	Plus/Minus	CP011369.1
IMP (Reverse Primer)	Pseudomonas aeruginosa strain PAER4_119, complete genome	317/319(99%)	1/319(0%)	Plus/Minus	CP013113.1
IMP (Forward Primer)	Pseudomonas aeruginosa strain PAER4_119, complete genome	316/320(99%)	3/320(0%)	Plus/Plus	CP013113.1

One UV mutant (labeled 42) was derived from parent CRPA strain (labeled 52, which harboring KPC, IMP and VIM genes). This mutant strain lost VIM gene, but preserved KPC and IMP genes according to PCR results. MIC and antibiotic sensitivity for carbapenem antibiotics were performed for this mutant and showed that carbapenem resistance was preserved. Selected PCR gene products were subjected to nucleotide sequencing for the parent strain 52 (PCR label 16) and its UV Mutant (strain 42).

Best Nucleotide alignment between PCR product sequences and gene bank data is shown in Table (5). VIM gene in the tested strain (Original parent strain label 52, PCR label 16) shows best alignment with *Pseudomonas aeruginosa* strain Ps-1 metallo-beta-lactamase VIM-11 gene, partial cds sequence and *Klebsiella pneumoniae* strain NRZ-28192 subclass B1 metallo-beta-lactamase VIM-46 (blaVIM) gene, blaVIM-46 allele, complete cds. KPC gene in the tested strain shows best alignment with *Pseudomonas aeruginosa* strain S04 90 genome. IMP gene in the tested strain shows best alignment with *Pseudomonas aeruginosa* strain PAER4_119, complete genome.

On the hand, KPC gene nucleotide sequence alignment between PCR product (Reverse primer) of PCR strain 16 (Parent strain 52) and UV mutant 42 shows 99% identity with 4 nucleotide difference. For forward primer, it shows 98% identical nucleotides and 9 nucleotide difference (Figs. 2 & 3).

IMP gene nucleotide sequence alignment between PCR product of PCR strain 16 (Parent strain 52) and UV mutant 42 shows 99% (3 nucleotide difference) and 98% (6 nucleotide difference) for reverse and forward primers respectively (Figs. 4 & 5).

Score	Expect	Identities	Gaps	Strand
1026 bits(555)	0.0()	564/568(99%)	2/568(0%)	Plus/Plus

```
Query 5   TCTGGATCTGATCTGGATTCCCATGGCGCTGCTGTATGCGCTGTGGATCCAGCAAAGGTT 64
          ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 6   TCTGGTTCTGATCTGGATTCCCATGGCGCTGCTGTATGCGCTGTGGATCCAGCAAAGGTT 65

Query 65  CGCCGCCAGCAGGCGCGGTGAAGATATCGCCGCGCCTTGAGCGCGGCGGTATCAGCGGTC 124
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 66  CGCCGCCAGCAGGCGCGGTGAAGATATCGCCGCGCCTTGAGCGCGGCGGTATCAGCGGTC 125

Query 125 AGGAGAAGAACAGCCGCGCAATGCCGCGGAAGGTCTTCTCGTTCCAGTACTTCAGCGGCA 184
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 126 AGGAGAAGAACAGCCGCGCAATGCCGCGGAAGGTCTTCTCGTTCCAGTACTTCAGCGGCA 185

Query 185 AGCCGGAGAGCAGCTCCCGGGCCAGCACCTTGTCCCGGTTCGAGCACTTGAGGAACATCG 244
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 186 AGCCGGAGAGCAGCTCCCGGGCCAGCACCTTGTCCCGGTTCGAGCACTTGAGGAACATCG 245

Query 245 AATTGCGGAAGCGCATGATCACCTGCTCGTAATCGGGGTGATCGCTGAACTGGCTGTACG 304
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 246 AATTGCGGAAGCGCATGATCACCTGCTCGTAATCGGGGTGATCGCTGAACTGGCTGTACG 305

Query 305 TCTTGAATACGTTGTCCACCATGAAACGGGCGTTCTTGTACGTATTGGTGGGATGCTTGC 364
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 306 TCTTGAATACGTTGTCCACCATGAAACGGGCGTTCTTGTACGTATTGGTGGGATGCTTGC 365

Query 365 GGTACTGGGCGAGGACCTCGCCAAGAATGTCAATGACGTAGCCAGCCTTGGTTACCGCCA 424
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 366 GGTACTGGGCGAGGACCTCGCCAAGAATGTCAATGACGTAGCCAGCCTTGGTTACCGCCA 425

Query 425 GTTCGATGTAGACATCTTCCAGGCGTATGTCCGGATCGAAACCGCCGACCTTCTCCAGGG 484
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 426 GTTCGATGTAGACATCTTCCAGGCGTATGTCCGGATCGAAACCGCCGACCTTCTCCAGGG 485
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Query 485 CTTCCCGCCGGAACATCAGGGTGGGCGCCATCGGGCCCGGCTTGCGGTCCAGGAACAGGT 544
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 486 CTTCCCGCCGGAACATCAGGGTGGGCGCCATCGGGCCCGGCTTGCGGTCCAGGAACAGGT 545

Query 545 CATCGAAGTCGAGACGG-GCAC-GGGGG 570
          |||||||||||||||| | || |||||
Sbjct 546 CATCGAAGTCGAGACGGCGTACAGGGGG 573
```

Fig. 2. KPC gene nucleotide sequence alignment between PCR product (Reverse primer) of PCR strain 16 (Parent strain 52) and UV mutant 42

Score	Expect	Identities	Gaps	Strand
1031 bits(558)	0.0()	578/587(98%)	3/587(0%)	Plus/Plus

```
Query 1   GGCTGAACTGATCTGGACGCAAGCCGGGCCCGATGGCGCCCACCCTGATGTTCCGGCGGG  60
          |||||||||| ||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1  GGCTGAACTGTTCTGGACGC-AGCCGGGCCCGATGGCGCCCACCCTGATGTTCCGGCGGG  59

Query 61  AAGCCCTGGAGAAGGTCGGCGGTTTCGATCCGGACATACGCCTGGAAGATGTCTACATCG  120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 60  AAGCCCTGGAGAAGGTCGGCGGTTTCGATCCGGACATACGCCTGGAAGATGTCTACATCG  119

Query 121 AACTGGCGGTAACCAAGGCTGGCTACGTCATTGACATTCTTGGCGAGGTCCTCGCCCAGT 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 120 AACTGGCGGTAACCAAGGCTGGCTACGTCATTGACATTCTTGGCGAGGTCCTCGCCCAGT 179

Query 181 ACCGCAAGCATCCCACCAATACGTACAAGAACGCCCGTTTCATGGTGGACAACGTATTCA 240
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 180 ACCGCAAGCATCCCACCAATACGTACAAGAACGCCCGTTTCATGGTGGACAACGTATTCA 239

Query 241 AGACGTACAGCCAGTTCAGCGATCACCCCGATTACGAGCAGGTGATCATGCGCTTCCGCA 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 240 AGACGTACAGCCAGTTCAGCGATCACCCCGATTACGAGCAGGTGATCATGCGCTTCCGCA 299

Query 301 ATTCGATGTTCCTCAAGTGCTCGAACCGGGACAAGGTGCTGGCCCGGGAGCTGCTCTCCG 360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 300 ATTCGATGTTCCTCAAGTGCTCGAACCGGGACAAGGTGCTGGCCCGGGAGCTGCTCTCCG 359

Query 361 GCTTGCCGCTGAAGTACTGGAACGAGAAGACCTTCCGCGGCATTGCGCGGCTGTTCTTCT 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 360 GCTTGCCGCTGAAGTACTGGAACGAGAAGACCTTCCGCGGCATTGCGCGGCTGTTCTTCT 419

Query 421 CCTGACCGCTGATACCGCCGCGCTCAAGGCGCGGCGATATCTTCACCGCGCCTGCTGGCG 480
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 420 CCTGACCGCTGATACCGCCGCGCTCAAGGCGCGGCGATATCTTCACCGCGCCTGCTGGCG 479

Query 481 GCGAACCTTTGCTGGATCCACAGCGCATACAGCAGCGCCATGGGAATCCAGATCAGGAAC 540
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 480 GCGAACCTTTGCTGGATCCACAGCGCATACAGCAGCGCCATGGGAATCCAGATCAGGAAC 539

Query 541 CAGTGTTCCTTGGGACGGGG-AGAGGGCCCCTT-aaaaaaaaaaaaa 585
          |||||||||||||||||||| ||| ||    || ||||||||||||
Sbjct 540 CAGTGTTCCTTGGGACGGGGCAGAAGGTTTTTTGAAAAAAAAAAAA 586
```

Fig. 3. KPC gene nucleotide sequence alignment between PCR product (Forward primer) of PCR strain 16 (Parent strain 52) and UV mutant 42

Score	Expect	Identities	Gaps	Strand
590 bits(319)	2e-173()	326/329(99%)	1/329(0%)	Plus/Plus

```
Query 9   AACGACGCGGGCCAGGTTGCACGCAGGCTGGGAGCCGCTCATGGACGCCGAGCTTATTCT 68
          ||||  |||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 8   AACGGCGCGGG-CAGGTTGCACGCAGGCTGGGAGCCGCTCATGGACGCCGAGCTTATTCT 66

Query 69  TAGTTGATTGAACGTTCAATAAAAACAAAATAGACTGGCCACCAATGACGTCGGTCGGTG 128
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 67  TAGTTGATTGAACGTTCAATAAAAACAAAATAGACTGGCCACCAATGACGTCGGTCGGTG 126

Query 129 CCCGCACGCACCTATCGACGAGAGGAGATAGCAATGCCCAAGGTCGGTATGCAGCCCATC 188
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 127 CCCGCACGCACCTATCGACGAGAGGAGATAGCAATGCCCAAGGTCGGTATGCAGCCCATC 186

Query 189 CGCCGCTCGCAACTGATTCACGCCACCCTGGAGGCGGTCGATCAGGTCGGCATGGGAGAC 248
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 187 CGCCGCTCGCAACTGATTCACGCCACCCTGGAGGCGGTCGATCAGGTCGGCATGGGAGAC 246

Query 249 GCCAGCATCGCCCTGATTGCCCGCCTGGCGGGTGTTTCCAACGGCATCATCAGCCACTAC 308
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 247 GCCAGCATCGCCCTGATTGCCCGCCTGGCGGGTGTTTCCAACGGCATCATCAGCCACTAC 306

Query 309 TTCCAGGACAAGAACCACCAAAACCATGA 337
          |||||||||||||||| ||||||||||||
Sbjct 307 TTCCAGGACAAGAACCCCCAAAACCATGA 335
```

Fig. 4. IMP gene nucleotide sequence alignment between PCR product (Reverse primer) of PCR strain 16 (Parent strain 52) and UV mutant 42

Score	Expect	Identities	Gaps	Strand
584 bits(316)	2e-171()	331/337(98%)	6/337(1%)	Plus/Plus

```
Query 8   GGCTGA-GA-GCCGTTGGAACACCCGCCAGGCGGGCAATCAGGGCGATGCTGGCGTCTCC 65
          ||||||  ||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 7   GGCTGATGATGCCGTTGGAACACCCGCCAGGCGGGCAATCAGGGCGATGCTGGCGTCTCC 66

Query 66  CATGCCGACCTGATCGACCGCCTCCAGGGTGGCGTGAATCAGTTGCGAGCGGCGGATGGG 125
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 67  CATGCCGACCTGATCGACCGCCTCCAGGGTGGCGTGAATCAGTTGCGAGCGGCGGATGGG 126

Query 126 CTGCATACCGACCTTGGGCATTGCTATCTCCTCTCGTCGATAGGTGCGTGCGGGCACCGA 185
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 127 CTGCATACCGACCTTGGGCATTGCTATCTCCTCTCGTCGATAGGTGCGTGCGGGCACCGA 186

Query 186 CCGACGTCATTGGTGGCCAGTCTATTTTGTTTTTATTGAACGTTCAATCAACTAAGAATA 245
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 187 CCGACGTCATTGGTGGCCAGTCTATTTTGTTTTTATTGAACGTTCAATCAACTAAGAATA 246

Query 246 AGCTCGGCGTCCATGAGCGGCTCCCAGCCTGCGTGCAACCTGGCCCGCGCCGTTCATCAC 305
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 247 AGCTCGGCGTCCATGAGCGGCTCCCAGCCTGCGTGCAACCTGGCCCGCGCCGTTCATCAC 306

Query 306 CTCGTCGCCAAAGTCCGCCC-AAATTATAGAAGAGTA 341
          |||||||||||||  ||||||  ||||||||  |||  |||
Sbjct 307 CTCGTCGCCAAAG-CCGCCCCAAATTATA-AAG-GTA 340
```

Fig. 5. IMP gene nucleotide sequence alignment between PCR product (Forward primer) of PCR strain 16 (Parent strain 52) and UV mutant 42

3.2 Discussion

Carbapenem antibiotics have been considered the last resort for treatment of infections due to the global spread of multidrug-resistant bacteria and the associated antibiotic resistance genes [27-30]. Molecular methods, including multiplex PCR and real time PCR were developed by many authors [31,32]. However, specific multiplex PCR to detect a set of carbapenemase encoding genes in one reaction was tested in the present study. This was restricted by the PCR product sizes and accordingly it was successful with the selected carbapenemase encoding genes IMP {PCR product: 488 bp}, VIM {PCR product: 280 bp}, SME {PCR product: 820 bp}. These results can facilitate and speed the detection of carbapenemase encoding genes that can be detected in few reactions instead of one reaction for each gene.

Selected CRPA strains that were previously induced for mutation by exposure to UV radiation (Parent strains 52, 57) were also treated with SDS and heat to enhance plasmid curing and the resultant strains were tested for antibiotic sensitivity and carbapenemase encoding genes. The carbapenem resistance and VIM gene (which was detected alone in the parent strain using verigene-nanosphere) were preserved after treatment. IMP and KPC genes that were previously detected by PCR in the parent strain were not detected (by verigene-nonosphere) before and after thermal or SDS treatment. Plasmid profiling was not performed before and after SDS and heat treatment as the resistance pattern did not change. Additionally, the preserved VIM has no confirmed or sole role in carbapenem resistance in the tested strain concluded after UV induced mutation as carbapenem resistance preserved after removal of VIM gene. KPC gene is carried only on a plasmid [33,34]. While IMP and VIM genes can be carried on plasmid or chromosome [35]. The current curing experiment plus the conclusions on this strain (from UV exposure experiment) are telling that, VIM gene was preserved and one or both of KPC and IMP were preserved and may be responsible for carbapenem resistance. But these data cannot exclude the role of non carbapenemase mechanisms in carbapenem resistance namely, low outer membrane permeability and efflux system [36,37]. Plasmid profiling and PCR detection of both carbapenemase and non carbapenemase encoding genes may provide better data regarding the role of plasmid associated genes in carbapenem resistance.

The sensitivity of the microarray based technique nanosphere-verigene was tested against PCR results in all the 12 strains harboring carbapenemase encoding genes. The result shows very low sensitivity or improper conditions of nanosphere-verigene technique in our study. Nansopshere-verigene results in the current study do not agree with other publication, who reported that 100% of known KPC genes were detected using nanosphere verigene BC-GN assay [38]. Ledeboer et al. 2015 showed that the PPA (positive percent agreement) for identification of resistance determinants was as follows: blaCTX-M,98.9%; blaKPC,100%; blaNDM,96.2%; blaOXA,94.3%; blaVIM,100%; andblaIMP,100% and all resistance determinant targets demonstrated >99.9% NPA (Negative percent agreement) [39]. However, the manufacturer instructions and publications are describing the technique as a rapid method for identification and detection of Gram positive and Gram negative bacteria and the associated resistance genes in positive blood cultures. In the present study, the method was modified to use this technique with the preserved bacterial strains which may affect the final results and accordingly improved testing conditions may be needed to achieve better performance and sensitivity. The main impact and advantage of nanosphere-verigene technology is the rapid detection and treatment response to bacteremia as concluded in a recent study, which reported that sub analysis demonstrated site-specific differences in the uptake of stewardship recommendations, whereby 32.4%, 50.5%, and 15.0% of cases at different hospitals demonstrated the expected change in antibiotics and suggested that Verigene had the expected impact in a third of the cases and the results reporting algorithm minimized the real-time involvement of the pharmacist while maintaining optimal patient management [40].

The alignments of PCR products for VIM, IMP and KPC genes support that these sequences of carbapenemase encoding genes are distributed among species of Gram negative bacteria. As an example, KPC gene is carried on plasmid only, as bla_{KPC} is in Tn440 which in turn is in plasmids, making these elements highly transferable and explaining their rapid dissemination in a hospital environment [33,34]. Sequencing of such genes provide a powerful evidence for its transfer as recently reported in

draft genome sequences of four blaKPC-containing bacteria identified as *Klebsiella aerogenes*, *Citrobacter freundii, and Citrobacter koseri*. Additionally, the draft genome sequence of a *K. aerogenes* strain did not originally contain a blaKPC gene, but was isolated from the patient who had the blaKPC-2 containing *K. aerogenes* strain [41].

On the other hand, there were little nucleotide differences between PCR products of the (tested) parent CRPA strain and its UV mutant regarding both KPC and IMP. It was not confirmed that these differences are due to exposure to UV radiation. In general, the capacity of prokaryotes to withstand significant UV radiation requires a wide array of physiological responses, including transcriptional regulation and cellular repair of irradiation-induced damage [42-44]. So, in induced mutation by UV exposure, VIM gene was lost, while IMP and KPC genes were preserved with no damage, repaired damage or little modification of their nucleotide sequence in the PCR amplified segments and the carbapenem resistance was not affected. Although, it was reported that little modifications by insertion or deletion of even one base pair of *oprD* gene can inactivate carbapenem resistance [45].

4. CONCLUSION

In conclusion, Multidrug-resistant Gram-negative bacteria such as *Acinetobacter* and *Pseudomonas* species are the main organisms spreading into the community. These organisms have the potential to spread resistance to other bacterial isolates into the community. Despite efforts to control carbapenem resistance, a definite solution to the problem is still far from achieving. Carbapenemase-encoding genes are already widespread in certain parts of the world, particularly, Europe, Asia and South America while the situation in other places such as sub-Saharan Africa is not well documented [46]. There is the need for active surveillance of carbapenemase-encoding genes as major step to controlling this worldwide challenge.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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