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ORIGINAL ARTICLE

Activity of imipenem/relebactam on *Klebsiella pneumoniae* with different mechanisms of imipenem non-susceptibility

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ABSTRACT

Background and Objectives: Imipenem/relebactam (IMP/R) is a newly FDA approved β-lactam/β-lactamase inhibitor combination. Relebactam ability to restore IMP activity could differ according to the cause of imipenem non-susceptibility. Therefore, we investigated the *in-vitro* activity of IMP/R against *Klebsiella pneumoniae* with different mechanisms of imipenem non-susceptibility.

Materials and Methods: Imipenem-nonsusceptible (IMP-NS) *K. pneumoniae* isolates were collected and characterized for β-lactamase encoding genes by multiplex PCR. For IMP-NS carbapenemase-negative isolates, study of *Ompk35* & *Ompk36* gene expression was performed by reverse transcription-PCR while efflux pump activity was studied by minimum inhibitory concentration (MIC) reduction assay using efflux pump inhibitor. Susceptibility testing of *K. pneumoniae* to IMP and IMP/R were achieved by broth microdilution (BMD) method.

Results: During the study period, 140 isolates of IMP-NS *K. pneumoniae* were collected. BMD method showed that relebactam restored IMP susceptibility in 100%, 60% and 49% of isolates that only harbor AmpC, extended spectrum beta lactamase (ESBL) and carbapenemases, respectively. IMP/R was most potent against all bla_{KPC} and 50% of bla_{OXA-48}–producing isolates. No demonstrable activity of IMP/R against *K. pneumoniae* harboring metallo-β-lactamases (MBLs). Out of 18 isolates with IMP non-suceptibility due to porins loss with overproduction of ESBL and/or AmpC, 14 (77.7%) isolates were IMP/R susceptible. IMP/R showed no activity against isolates with only efflux pump hyperactivity.

Conclusion: Relebactam could restore IPM activity in KPC or AmpC-producing IMP/NS *K. pneumoniae* but with no activity against MBL^- producing isolates. Relebactam activity against isolates harbouring-bla_{OXA-48} or with altered *Ompk35* & *Ompk36* gene expression and efflux pump hyperactivity need further studies. Therefore, using IMP/R antibiotic in the treatment of infections caused by IMP/NS *K. pneumoniae* should be based on its molecular profile of IMP resistance to optimize the utility of IMP/R.

Keywords: Beta lactamases; *Klebsiella pneumoniae*; Polymerase chain reaction; Imipenem; Relebactam

INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is one of the main causes of either hospital or community-acquired infections. It causes a wide variety of infections including; infections of respiratory tract, urinary tract, blood stream and intra-abdominal abscesses (1).

For a long time, carbapenems have been the last antibiotic choice for the management of patients infected with extended spectrum beta lactamase (ESBL)-producing *K. pneumoniae* (2). Unfortunately,

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the widespread use of antibiotics, its self-administration by patients and non-implementation of policies for proper and wise use of antibiotics in hospitals lead to emergence of carbapenem-resistant *K. pneumoniae* (CRKP) strains which are spreading worldwide (3, 4).

Non-susceptibility of *K. pneumoniae* to carbapenems is mediated by a variety of mechanisms including production of one or more of carbapenemase enzymes, impaired permeability of outer membrane together with hyperproduction of β-lactamases (ESBL or AmpC), and antibiotic efflux across the outer membrane (5).

The rapid rise of carbapenem resistance among *K. pneumoniae* together with the availability of only few choices of antimicrobial therapy for treating infections caused by CRKP has led to the development of new antimicrobial agents to solve this problem. One of these antibiotics is imipenem/relebactam (IMP/R) (6).

Relebactam is a bicyclic diazabicyclooctane new β-lactamase inhibitor. Recently, in July 2019, IMP/R obtained approval by the Food and Drug Administration (FDA) for the treatment of complicated urinary tract and intra-abdominal infections (7). The ability of this recently developed antibiotic combination to restore activity of imipenem (IMP) could vary with the mechanism causing IMP non-susceptibility. Therefore, we aimed to elucidate to what extent the IMP/R combination can restore IMP susceptibility for a collection of IMP-NS *K. pneumoniae* isolates according to their mechanisms of IMP non-susceptibility. This study was approved from IRB committee in our faculty of medicine, Mansoura University.

MATERIALS AND METHODS

Bacterial isolates. Clinical samples were collected from infected patients hospitalized in Mansoura University hospitals in the period from November 2017 to April 2019. Processing of all samples was done in microbiology laboratory of Specialized Medical Hospital at Mansoura University and in medical microbiology and immunology department at Mansoura faculty of medicine. Identification of organisms up to species level and preliminary determination of IMP minimum inhibitory concentration (MIC) were done by automated Vitek®2 compact system (bioMérieux, Marcy-l'Etoile, France).

The mechanism of IMP non-susceptibility among *K. pneumoniae* with IMP MIC ≥ 2 µg/ mL was determined firstly by genotypic detection of the commonest genes encoding carabapenemases, ESBLs and AmpC. The overproduction of ESBL and/or AmpC together with loss of porins could be responsible for carbapeneme non-susceptibility in isolates that harbor genes for ESBL and/or AmpC but are free of carbapenemase genes (8). Therefore, the mechanism of IMP non-susceptibility among carbapenemase-free isolates was investigated by analysis of gene expression of outer membrane proteins (*Ompk35* & *Ompk36*), measurement of the efflux pump activity and phenotypic assays of the ESBL and/or AmpC overproduction.

Phenotypic assays of ESBL and AmpC enzymes. Phenotypic detection of ESBL was done by Vitek2 system using AST-GN73 cards. AmpC overproduction was done by cefoxitin-cloxacillin combined disk test using discs of cefoxitin (30 µg) with and without cloxacillin (200 µg) as inhibitor of AmpC β-lactamase. AmpC production was considered if the inhibition zone of cefoxitin with cloxacillin disc was increased ≥ 4 mm than the inhibition zone of cefoxitin disk alone (9).

Molecular detection of β–lactamase genes. DNA was extracted from all IMP-NS *K. pneumoniae* isolates by DNA extraction kits (QIAGEN, GmbH, Germany) according to manufacture instructions. Three sets of multiplex PCR assays were done to detect the most common genes of Carbapenemases including bla_{KPC}, bla_{OXA48} and metallo-β-lactamases (bla_{NDM}, bla_{IMP}, bla_{VIM}), ESBL (bla_{CTXM}, bla_{TEM}, bla_{SHV}) and AmpC (MOX, CIT, DHA, ACC, EBC and FOX). Primers used for detection of all investigated genes are listed in Table 1. Amplification of the investigated genes was performed following protocols previously described by Poirel et al. for carbapenemase genes (10), by Lee et al. for ESBL genes (11) and by Pérez-Pérez and Hanson for AmpC genes (12).

In brief, each multiplex PCR was performed in a total reaction volume of 25 µL that contains a hot start Taq DNA polymerase master mix (12.5 µL), DNA template adjusted to 50 ng/µl (2 µL), forward and reverse primer pairs specific for each analyzed gene (variable concentrations following the reference protocol) and nuclease free water (up to a total volume of 25 µL). Amplification was done in a thermal

Table 1. Sequence of primers used in multiplex PCR

Target gene	Primer sequence (5′–3′)	Product size (bp)
16S rRNA	F:AATACCGCATAATGTCGC	622
	R:CCCTCGTTTGTCCATATCT	
OmpK35	F:GCGTCCAATGTTGAAGGT	778
	R: TAATGTGGTTTCGCCGAC	
OmpK36	F:CCGTAACTCTGATTTCTTCG	588
	R:TTAGTTGGACGACCTGCT	
bla_{IMP}	F:GGAATAGAGTGGCTTAAYTCTC	232
	R:GGTTTAAYAAAACAACCACC	
bla_{VIM}	F:GATGGTGTTTGGTCGCATA	390
	R:CGAATGCGCAGCACCAG	
bla_{NDM}	F:GGTTTGGCGATCTGGTTTTC	621
	R:CGGAATGGCTCATCACGATC	
bla_{OXA-48}	F:GCGTGGTTAAGGATGAACAC	438
	R:CATCAAGTTCAACCCAACCG	
bla_{KPC}	F:GTCTAGTTCTGCTGTCTTG	798
	R:CTTGTCATCCTTGTTAGGCG	
bla_{TEM}	F:GAGACAATAACCCTGGTAAAT	851
	R:AGAAGTAAGTTGGCAGCAGTG	
bla_{CTX}	F:GAAGGTCATCAAGAAGGTGCG	550
	R:GCATTGCCACGCTTTTCATAG	
bla_{SHV}	F:AAGATCCACTATCGCCAGCAG	231
	R:ATTCAGTTCCGTTTCCCAGCGG	
bla_{MOX}	F:GCT GCT CAA GGA GCA CAG GAT	520
	R:CAC ATT GAC ATA GGT GTG GTG C	
bla_{CIT}	F:TGG CCA GAA CTG ACA GGC AAA	462
	R:TTT CTC CTG AAC GTG GCT GGC	
bla_{DHA}	F:AAC TTT CAC AGG TGT GCT GGG	405
	R:CCG TAC GCA TAC TGG CTT TGC	
bla_{ACC}	F:AAC AGC CTC AGC AGC CGG TTA	346
	R:TTC GCC GCA ATC ATC CCT AGC	
bla_{EBC}	F:TCG GTA AAG CCG ATG TTG CGG	302
	R:CTT CCA CTG CGG CTG CCA GTT	
bla_{FOX}	F:AAC ATG GGG TAT CAG GGA GAT G	190
	R:CAA AGC GCG TAA CCG GAT TGG	

F=Forward; R= Reverse; D=AorGorT; Y=CorT.

cylcler (Applied Biosystems) according to conditions previously described. The optimal annealing temperature was 52ºC for carbapenemase genes, 62ºC for ESBL genes and 64ºC for AmpC genes. Detection of the amplified DNA was done by electrophoresis using agarose gel stained with ethidium bromide at 100V for 1 h in 1× TAE buffer. The separated bands were visualized by UV transillmuinator using 100 bp and 50 bp DNA ladder.

Determination of efflux pump activity. Efflux activity was detected by MIC reduction assay using efflux pump inhibitor; carbonyl-cyanide3-chlorophenyl-hydrazine (CCCP) (HiMedia). IMP MIC of the tested isolate was determined by agar dilution method on Mueller-Hinton agar that contain 20 µg/mL of CCCP and compared to its MIC in the absence of CCCP. Significant pump activity with possible efflux pump gene overexpression is considered if there is a fourfold reduction in MIC of IMP in presence of CCCP (13).

Analysis of outer membrane proteins (*Ompk35* & *Ompk36*) gene expression. Gene expression analysis was performed by reverse transcription-PCR (RT-PCR) using OneStep RT-PCR Kit (QIAGEN). Extraction of RNA was done using an RNeasy Protect Bacteria MiniKit (QIAGEN) according to the manufacturer's instructions. Normalization of mRNA expression levels in different strains was performed by 16S rRNA. Reverse transcription was done at 50ºC for 30 min, then initial PCR activation achieved at 95ºC for 15 min. DNA amplification cycles included; denaturation at 94ºC (1 min), then annealing at 48ºC (1 min) followed by extension at 72ºC (1 min). Cycles was repeated 40 times, thereafter, the final extension lasted for 10min at 72ºC (14). The carbapenem-susceptible *K. pneumoniae* ATCC 13883 (wild-type *OmpK35* and *OmpK36*) was used as a control strain.

IMP and IMP/R susceptibility testing. Susceptibility of all *K. pneumoniae* isolates to both IMP and IMP/R with determination of MIC was performed by broth microdilution (BMD) method simultaneously on the same day according to CLSI recommendations (15). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used as for quality control. Imipenem and relebactam powder were purchased from Sigma-Aldrich (St. Louis, USA). Doubling dilutions of IMP starting from 0.03 to 128 µg/mL with a relebactam fixed at concentration of 4 µg/Ml were used. MIC interpretation of both IMP and IMP/R was done according to CLSI guidelines (15). For IMP, *K. pneumoniae* was considered susceptible, intermediate and resistant if with MIC ≤1 µg/Ml, equal 2 µg/mL and ≥4 µg/mL, respectively. For IMP/R, *K. pneumoniae* was considered susceptible, intermediate and resistant if with MIC ≤1/4, equal 2/4 and ≥4/4, respectively.

Data analysis. Statistical analysis was done using Statistical Package for Social Sciences (SPSS, version 22; Chicago, IL, USA). Categorical variables were described in numbers and percentages.

RESULTS

One hundred and forty of IMP-NS *K. pneumoniae* isolates were collected from bacterial cultures. These isolates were obtained from cultures of urine (n=67), blood (n=54) and ascetic fluid (n=19).

Beta-lactamase profile of IMP/NS *K. pneumoniae*. Multiplex PCR analysis categorized the studied IMP-NS isolates into 120 (85.7%) carbapenemase producers and 20 (14.3%) non-carbapenemase producers. Carbapenemases were detected either alone in 102 (72.8%) isolates or in combination with other β-lactamases in 18 (12.8%) isolates; 12 (8.5%) isolates co-produced also ESBLs and 6 (4.3%) isolates harbored also both ESBLs and AmpC. The most common detected carbapenemase gene was bla_{KPC} (n=58,41.4%); detected either alone in 44 (31.4%) isolates or together with other carbenemases in 14 (10%) isolates. MBL genes (bla_{NDM}, bla_{IMP}, bla_{VIM}) were detected in 48 (34.3%) isolates; alone in 36 (25.7%) isolates and in combination with other carbapenemases in 12 (8.6%) isolates. The least prevalent carbapenemase gene was bla_{OXA-48}; found alone in 8 (5.7%) isolates and coproduced with other cabapenemase in 6 (4.3%) isolates. The twenty carbapenemase non-producer isolates were categorized as 10 (7.1%) ESBL-only producers, 6 (4.3%) AmpC-only producers and 4 (2.9%) co-producers of both ESBL and AmpC Table 2.

Porins expression and efflux pump activity in carbapenemase-negative IMP-NS isolates. Expression loss of *OmpK35* and/or *OmpK36* in combination with production of ESBL and/or AmpC was detected in 18/20 (90%) of carbapenemase-negative isolates. Out of ESBL-only producers, 8 (80%) showed expression loss of *OmpK35* and/or *OmpK36* without efflux pump hyperactivity while the remaining two isolates showed only efflux pump hyperactivity without any expression loss of outer membrane proteins. All six AmpC-only producers showed expression loss of both *OmpK35* and *OmpK36* and none of them showed efflux pump hyperactivity. All four ESBL and AmpC coproducers showed expression loss of *OmpK35* and/or *OmpK36* without efflux pump hyperactivity with no demonstrable efflux pump hyperactivity, Table 3.

***In vitro* activity of IMP and IMP/R by BMD method in relation to mechanism of IMP non-susceptibility.** Out of the collected 140 IMP/NS *K. pneumoniae* isolates; 14 (10%) showed intermediate susceptibility to IMP and 126 (90%) were IMP resistant with MIC ranged from 4 to 128 µg/mL. Addition of relebactam restored susceptibility to IMP in 64 (45.7%) of all IMP-NS *K. pneumoniae* isolates with reduction of MIC_{50} 8 folds from 16 to 2 µg/mL and lowering of MIC_{90} 2 folds from 64 to 32 µg/mL.

In accordance to β-lactamase type, relebactam restored IMP activity in 50/102 (49%) of carbapenemase-only producing isolates, in 6/6 (100%) of AmpC-only producing isolates, in 6/10 (60%) of ESBL-only producing isolates and in 50% of isolates co-producing both ESBL & AmpC. However, none of isolates co-producing carbapenemase in combination with either ESBL alone or with both ESBL and AmpC rendered susceptible to IMP by relebactam, Table 2.

Regarding the effect of carbapenemase type on the activity of relebactam, addition of relebactam restored IMP susceptibility in 100% of KPC-producers, in 50% of OXA-48- producers and in 100% of isolates harboring both KPC and OXA-48 enzymes. However, none of isolates harboring MBL enzymes either alone or in combination with either bla_{KPC} or bla_{OXA-48} rendered susceptible to IMP by addition of relebactam. Reduction of MIC_{90} was 64 folds in bla_{KPC} producers and 32 folds in isolates producing bla_{OXA-48} either alone or in combination with bla_{KPC} (Table 2).

For the *in vitro* effect of IMP/R on IMP-NS carbapenemase-negative *K. pneumoniae*, none of the two isolates with only efflux pump hyperactivity rendered IMP susceptible by addition of relebactam. Out of 18 isolates with expression loss of *OmpK35* and/or *OmpK36* in combination with production of ESBL and/or AmpC, IMP susceptibility was restored in 14 (77.7%) of them. These IMP/R susceptible isolates included; all AmpC-only producers, 6/8 (75%) of ESBL-only producers, 2 (50%) of ESBL and AmpC co-producers, (Table 3).

DISCUSSION

Many studies investigated the *in-vitro* activity of IMP/R against carbapenemase mediated IMP

Table 2. MIC distribution of imipenem and imipenem with relebactam in relation to β- lactamase type in *K. pneumoniae* isolates

B- lactamase type (n)	AB	No of isolates (cumulative %) inhibited at MIC (µg/mL)													MIC_{50}	MIC_{90}	S%
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128			
Carbapenemase positive (120)	I	0	0	0	0	0	0	10 (8.3)	16 (21.7)	22 (40)	24 (60)	22 (78.3)	16 (91.7)	10 (100)	16	64	0
	I/R	16 (13.3)	12 (23.3)	14 (35)	0	4 (38.3)	4 (41.7)	6 (46.7)	10 (55)	14 (66.7)	16 (80)	14 (91.7)	10 (100)	0	4	32	41.7
Carbapenemase-only producers (102)	I	0	0	0	0	0	0	10 (9.8)	16 (25.5)	20 (45.1)	20 (64.7)	16 (80.4)	14 (94.1)	6 (100)	16	64	0
	I/R	16 (15.7)	12 (27.5)	14 (41.2)	0	4 (45.1)	4 (49)	6 (54.9)	10 (64.7)	10 (74.5)	12 (86.3)	8 (94.1)	6 (100)	0	2	32	49
bla_{KPC} only producers (44)	I	0	0	0	0	0	0	8 (18.2)	8 (36.4)	10 (59.1)	8 (77.3)	6 (90.9)	2 (95.5)	2 (100)	8	32	0
	I/R	16 (36.4)	10 (59.1)	12 (86.4)	0	2 (90.9)	4 (100)	0	0	0	0	0	0	0	0.06	0.5	100
MBL only producers (36)	I	0	0	0	0	0	0	0	8 (22.2)	8 (44.4)	10 (72.2)	4 (83.3)	6 (100)	0	16	64	0
	I/R	0	0	0	0	0	0	0	8 (22.2)	8 (44.4)	10 (72.2)	4 (83.3)	6 (100)	0	16	64	0
bla_{OXA-48} producers (8)	I	0	0	0	0	0	0	2 (25)	0	2 (50)	0	2 (75)	0	2 (100)	8	128	0
	I/R	0	2 (25)	2 (50)	0	0	0	0	0	0	2 (75)	2 (100)	0	0	0.12	32	50
bla_{KPC} & bla_{OXA-48} coproducers (2)	I	0	0	0	0	0	0	0	0	0	2 (100)	0	0	0	16	16	0
	I/R	0	0	0	0	2 (100)	0	0	0	0	0	0	0	0	0.5	0.5	100
bla_{KPC} & MBL coproducers (8)	I	0	0	0	0	0	0	0	0	0	0	4 (50)	4 (100)	0	32	64	0
	I/R	0	0	0	0	0	0	2 (25)	0	2 (50)	0	4 (100)	0	0	8	32	0
bla_{KPC} & MBL & bla_{OXA-48} coproducers (4)	I	0	0	0	0	0	0	0	0	0	0	0	2 (50)	2 (100)	64	128	0
	I/R	0	0	0	0	0	0	2 (50)	0	0	2 (100)	0	0	0	2	16	0
ESBL & Carbapenemase coproducers (12)	I	0	0	0	0	0	0	0	0	2 (16.7)	4 (50)	6 (100)	0	0	16	32	0
	I/R	0	0	0	0	0	0	0	0	4 (33.3)	4 (66.7)	4 (100)	0	0	16	32	0
ESBL & AmpC & Carbapenemase coproducers (6)	I	0	0	0	0	0	0	0	0	0	0	0	2 (33.3)	4 (100)	128	128	0
	I/R	0	0	0	0	0	0	0	0	0	0	2 (33.3)	4 (100)	0	64	64	0
Carbapenemase negative (20)	I	0	0	0	0	0	0	4 (20)	0	4 (40)	4 (60)	0	6 (90)	2 (100)	16	64	0
	I/R	2 (10)	2 (20)	0	2 (30)	2 (40)	6 (70)	4 (90)	2 (100)	0	0	0	0	0	1	2	60
ESBL only producers (10)	I	0	0	0	0	0	0	2 (2)	0	2 (40)	2 (60)	0	4 (100)	0	16	64	0
	I /R	0	0	0	0	2 (20)	4 (60)	2 (80)	2 (100)	0	0	0	0	0	1	4	60
AmpC only producers (6)	I	0	0	0	0	0		2 (33.3)		2 (66.7)	2 (100)	0	0	0	8	16	0
	I /R	2 (33.3)	2 (66.7)	0	2 (100)	0	0	0	0	0	0	0	0	0	0.06	0.25	100
ESBL & AmpC coproducers (4)	I	0	0	0	0	0	0	0	0	0	0	0	0	2 (100)	64	128	0
	I /R	0	0	0	0	0	2 (50)	2 (100)	0	0	0	0	22 (91.4)	12 (100)	1	2	50
Total isolates (140)	I	0	0	0	0	0	0	14 (10)	16 (21.4)	26 (40.0)	28 (60.0)	22 (75.7)	10 (100)	0	16	64	0
							10 (45.7)	10 (52.9)	12 (61.4)	14 (71.4)					2	32	45.7

AB= Antibiotic; I= Imipenem alone; I/R= Imipenem with relebactam; S=Susceptible; MIC= Minimal inhibitory concentration; MBL= Metallo-β-latamase, ESBL= Extended-spectrum-β-lactamase.

Table 3. Effect of relebactam on susceptibility to IMP in carbapenemase-negative IMP-resistnt *K. pneumoniae* isolates in relation to ESBL & AmpC overproduction, expression of porin genes and efflux pump activity.

Isolate No	Phenotypic detection of		Gene expression of		Efflux activity	MIC (µg/mL)	
	ESBL	AmpC	*OmpK35*	*OmpK36*		IMP	IMP/R
7	+	-	Lost	Present	No	2	0.5
16	-	+	Lost	Lost	No	2	0.03
24	+	+	Lost	Present	No	64	1
35	+	-	Lost	Lost	No	16	1
40	-	+	Lost	Lost	No	2	0.03
42	+	-	Present	Present	Yes	64	4
50	-	+	Lost	Lost	No	16	0.25
55	+	-	Lost	Present	No	2	0.5
60	+	-	Lost	Lost	No	64	2
64	+	-	Lost	Present	No	8	1
69	+	+	Lost	Lost	No	128	2
72	-	+	Lost	Lost	No	8	0.06
80	+	-	Lost	Present	No	8	1
89	-	+	Lost	Lost	No	16	0.25
102	+	-	Lost	Lost	No	64	2
115	-	+	Lost	Lost	No	8	0.06
120	+	+	Lost	Present	No	64	1
129	+	-	Lost	Lost	No	16	1
132	+	+	Lost	Lost	No	128	2
138	+	-	Present	Present	Yes	64	4

IMP= Imipenem alone; IMP/R= Imipenem with relebactam; MIC= Minimal inhibitory concentration, ESBL= Extended-spectrum-β-lactamase

non-susceptibility in Gram-negative bacilli. However, only limited studies investigated the in-vitro activity of this new antibiotic against different mechanisms of carbapenem non-susceptibility in *K. pneumoniae*. We compared the in-vitro effect of IMP/R versus IMP alone in a collection of IMP-NS *K. pneumoniae* isolates harboring different mechanisms of IMP non-susceptibility.

Multiplex PCR performed for detection of the type of β-lactamases responsible for the reduced IMP susceptibility among the collected isolates revealed that IMP non-susceptibility are mediated by carbapeneamses in 120 (85.7%) of isolates. Whereas the IMP non-susceptibility in the remaining 20 (14.3%) isolates are mediated by mechanisms other than carbapenemases. This is similar to Gomez-Simmonds et al. who reported that 88.9% and 10.4% of their carbapenam resistant *Enterobactericeae* are carbapenemase positive and negative, respectively (16).

To date of performing this analysis, susceptibility breakpoints of IMP/R were not established by CLSI 2019. Therefore, breakpoints of IMP published in CLSI 2019 were used for interpretation of both IMP and IMP/R susceptibility results. However, CLSI published in March 2021 the interpretive criteria of IMP/R by BMD method (17). Fortunately, these recently published criteria are the same as we used in the interpretation, so it did not affected on our findings.

In the present study, the most potent activity of IMP/R was against bla_{KPC}-carrying isolates. Relebactam restored susceptibility to IMP in 100% of the isolates that harbor carbapenemase of bla_{KPC}-type. This agrees with other studies reporting that relebactam restored imipenem susceptibility in all *K. pneumoniae* with carbapenemases of bla_{KPC-2} and bla_{KPC-3} typee (18).

On the other hand, addition of relebactam did not restore IMP susceptibility in any of MBL-only producing *K. pneumoniae* isolates. This is consistent with previous study that evaluated IMP/R susceptibility among 113 isolates of *K. pneumoniae* harbor-

ing class B-metallo-β-lactamases, and found that none of them rendered IMP susceptible by relebactam regardless of MβL type (6).

Regarding the effect of IMP/R on the bla_{OXA-48}-producing *K. pneumoniae* isolates, half of the evaluated IMP-NS bla_{OXA-48} producers were found to be susceptible to IMP/R. This is in accordance with Schmidt-Malan et al. who found that 50% of their bla_{OXA-48}-positive isolates had IMP/R MICs of 1 µg/mL (19). However, other studies reported that IMP/R has no activities against isolates with class D-carbapenemase (7, 18). Therefore, it seems that relebactam does not consistently inhibit IMP in isolates producing bla_{OXA-48}-carbapenemases.

In our study, investigation of porins gene expression, efflux pump as well as ESBL and AmpC overproduction in carbapenemase-negative IMP non-susceptible isolates showed that IMP non-susceptibility in 90% of these isolates could be caused by combined loss of either one or both porins with overproduction of ESBL/or AmpC. This is in line with Hamzaoui et al. who proved that loss of porins in *K. pneumoniae* can extend resistance spectrum mediated either by ESBLs or by plasmid-mediated AmpC enzymes to include carbapenems as well (8). This could be attributed to a residual capacity of ESBLs and AmpC enzymes to hydrolyse carbapenems, albeit at a very low efficiency (20).

Regarding the impact of relebactam on non-susceptibility to IMP caused by overproduction of ESBL and/or AmpC with loss or alteration of outer membrane proteins, we found that all AmpC-only producers, 75% of ESBL-only producers and 50% of ESBL and AmpC coproducers rendered susceptible to IMP by addition of relebactam. Similarly, Haidar et al. demonstrated a modest potentiation of IMP activity against carbapenem-resistant *Enterobacteriaceae* isolates producing ESBL and/or AmpC enzymes with membrane impermeability by addition of relebactam (21). Also, Gomez-Simmonds et al. found that 88% of their carbapenemase-negative *Enterobactericeae* carrying either bla_{AmpC} or $bla_{CTX-M-15}$ genes rendered IMP susceptible by relebactam (16). Moreover, relebactam has been found previously to restore the *in-vitro* activity of imipenem activity against *P. aeruginosa* that are carbapenem-resistant due to impermeability arising from porins loss combined with AmpC expression (6).

However, we observed that relebactam could not decrease IMP MIC to a susceptible level in six carbapenemase-negative isolates. Two of these isolates were ESBL-only producers expressing both porins but with efflux pump activity. Since relebactam is not subject to efflux (22), therefore, IMP/R resistance in these two isolates could be due to functional mutations in major *OmpK36* porin (21). The other four isolates that showed non-susceptibility to IMP/R (MIC=2) were two ESBL-only producers and two coproducers of AmpC and ESBL showing expression loss of both porins. Therefore, it is possible that these isolates is expressing ESBL or AmpC in high amount so that relebactam is unable to render these isolates susceptible to imipenem (22).

The overall rate of IMP/R activity against IMP-NS *K. pneumoniae* in our study was low (45.7%) as compared to other studies that demonstrated higher activity of IMP/R against *K. pneumoniae* with rates of susceptibility exceeding 95% (16). This could be attributed to inclusion among the collected isolates in this study of considerably high percentage (34.3%) of *K. pneumoniae* isolates expressing β-lactamases, as MBLs, that not inhibited by relebactam.

CONCLUSION

Efficacy of relebactam in rendering IMP/NS *K. pneumoniae* susceptible to IMP varied according to the mechanism of IMP non-susceptibility. It had the highest activity on KPC carbapenemase and AmpC β-lactamse. However, it showed no activity on MBL genes (bla_{NDM}, bla_{IMP}, bla_{VIM}) and efflux pump overactivity. Rlebactam activity in IMP/NS *K. pneumoniae* with production of bla_{OXA-48} or with altered membrane permeability needs more study.

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