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 carbapenem-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*<sub>KPC</sub> and 50% of *bla*<sub>ESBLs</sub> producing isolates. No demonstrable activity of IMP/R against *K. pneumoniae* harboring metallo-β-lactamases (MBLs). Out of 18 isolates with IMP non-susceptibility 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 IMp activity in KPC or AmpC-producing IMP/NS *K. pneumoniae* but with no activity against MBL producing isolates. Relebactam activity against isolates harbouring-<i>bla</i><sub>ESBL</sub> 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,
the widespread use of antibiotics, its self-administra-
tion by patients and non-implementation of policies
for proper and wise use of antibiotics in hospitals
lead to emergence of carbapenem-resistant K. pneu-
moniae (CRKP) strains which are spreading world-
wide (3, 4).

Non-susceptibility of K. pneumoniae to carba-
penems is mediated by a variety of mechanisms in-
cluding 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 in-
fected caused by CRKP has led to the develop-
ment 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 Administra-
tion (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-suscepti-
bility. 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 Uni-
versity hospitals in the period from November 2017
to April 2019. Processing of all samples was done in
microbiology laboratory of Specialized Medical Hos-
pital at Mansoura University and in medical micro-
biology 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 deter-
mined firstly by genotypic detection of the common-
est genes encoding carbapenemases, 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 har-
bor genes for ESBL and/or AmpC but are free of car-
bapenemase genes (8). Therefore, the mechanism of
IMP non-susceptibility among carbapenemase-free
isolates was investigated by analysis of gene ex-
pression 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 overproduc-
tion was done by cefoxitin-cloxacillin combined disk
test using discs of cefoxitin (30 μg) with and with-
out cloxacillin (200 μg) as inhibitor of AmpC β-lac-
tamase. AmpC production was considered if the in-
hibition zone of cefoxitin with cloxacillin disc was
increased ≥ 4 mm than the inhibition zone of cefoxi-
tin disk alone (9).

Molecular detection of β-lactamase genes. DNA
was extracted from all IMP-NS K. pneumoniae iso-
lates 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 blaKPC, blaOXA48 and metallo-β-lactamases
(blaNDM, blaIMP, blaVIM), ESBL (blaCTXM, blaTEM,
blaSHV) 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 in-
vestigated genes was performed following protocols
previously described by Poirel et al. for carbapene-
mase 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 vol-
ume 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:AACTACGCTATGTCGCA     | 622              |
|             | R:CCCTCCTTGCATATCTC     |                  |
| OmpK35      | F:GCATCAAGTGGGATGAGCT  | 778              |
|             | R:TAATGTTGTTGCACGACG   |                  |
| OmpK36      | F:CCTAAGTGGGATGAGCT    | 588              |
|             | R:TTAGTTGACGACCTCGT    |                  |
| blaIMP      | F:GGAAATAGGAGGCTTAATCTC | 232             |
|             | R:GTTTAAAYAAAAGACACACGC|                  |
| blaVIM      | F:GAGGCTGTTCTGTCACAATA | 390              |
|             | R:GCGATGCGAGCGACGCAG   |                  |
| blaNDM      | F:GCCTTGCAGTGTTTTC     | 621              |
|             | R:GCGATGCGAGCGACGCAG   |                  |
| blaOXA46    | F:GCTAATCTGACCTCAAC    | 438              |
|             | R:GCTGTTGATGGATGATGAC  |                  |
| blaKPC      | F:GCTAATCTGACCTCAAC    | 798              |
|             | R:GCTGTTGATGGATGATGAC  |                  |
| blaTEM      | F:GAGGCTGTTCTGTCACAATA | 851              |
|             | R:GAGGTAAGGGGCGAGCGAG  |                  |
| blaCTX      | F:GCTATGCATCAAGGATGTCG | 550              |
|             | R:GCATTGCCAGCTTTTATAG  |                  |
| blaNDV      | F:GATTGTCACACTAGCATC   | 231              |
|             | R:ATTGATGTCACACTAGCATC |                  |
| blaMOX      | F:GCTGCTCAAGGACGCTGAG  | 520              |
|             | R:GACATGACATGCTGACGCTA |                  |
| blaCIT      | F:TGGCCCACTTCTGACGCTAA | 462              |
|             | R:TCTATTCTGACGCTGACGCTAA |                |
| blaOXA      | F:AATCTGCTTGATTGCTGATG | 405              |
|             | R:CCCTGCGATGCTGCTGCTG  |                  |
| blaACC      | F:GAGGCTGTTCTGTCACAATA | 346              |
|             | R:GCTGTTGATGGATGATGAC  |                  |
| blaABC      | F:GCTGCTCAAGGACGCTGAG  | 302              |
|             | R:CTTGCTTGACGCTGACGCTG |                  |
| blaFOX      | F:AATCTGCTTGATTGCTGATG  | 190              |
|             | R:CCCTGCGATGCTGCTGCTG  |                  |

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

cycler (Applied Biosystems) according to conditions previously described. The optimal annealing temperature was 52°C for carbapenemase genes, 62°C for ESBL genes and 64°C 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 1x TAE buffer. The separated bands were visualized by UV transilluminator 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-chloro-phenyl-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 a RNAse 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 50°C for 30 min, then initial PCR activation achieved at 95°C for 15 min. DNA amplification cycles included; denaturation at 94°C (1 min), then annealing at 48°C (1 min) followed by extension at 72°C (1 min). Cycles was repeated 40 times, thereafter, the final extension lasted for 10 min at 72°C (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 a quality control. Imipenem and relebactam powder was 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 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 blaKPC(n=58,41.4%); detected either alone in 44 (31.4%) isolates or together with other carbapenemases in 14 (10%) isolates. MBL genes (blaNDM,blaIMP,blalmpK3) 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 blaoxa-48 found in 8(5.7%) isolates and co-produced with other carbapenemase in 6 (4.3%) isolates. The twenty carbapenem 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 MICso 8 folds from 16 to 2 µg/mL and lowering of MICso 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 blakPC or blaoxa-48 rendered susceptible to IMP by addition of relebactam. Reduction of MICso was 64 folds in blakPC producers and 32 folds in isolates producing blaoxa-48 either alone or in combination with blakPC(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
The table shows the activity of imipenem against various strains of bacteria.

### Table 2: MIC Distribution of Imipenem

| Antibiotic | MIC (µg/ml) | No. of Strains | Active (%) |
|------------|-------------|----------------|------------|
| Imipenem   | 0.125-2.0   | 20             | 90.9%      |

**Legend:**
- **MIC:** Minimum Inhibitory Concentration
- **AB:** Antibiotic
- **% Active:** Percentage of strains that are active against the antibiotic

**Notes:**
- The MIC values are cumulative from all strains tested.
- The data is presented in a table format with columns for Antibiotic, MIC, No. of Strains, and Active (%).
- The MIC values range from 0.125 to 2.0 µg/ml.
- The percentage of active strains ranges from 79.1% to 100%.
- The table includes a total of 20 strains tested.

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**Additional Information:**
- The study was conducted using a panel of 20 strains of bacteria.
- The MIC values were determined using a standard methodology.
- The results indicate varying levels of sensitivity to imipenem among the tested strains.

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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 carbapenamases 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 Enterobacteriaceae 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 blaKPC-carrying isolates. Relebactam restored susceptibility to IMP in 100% of the isolates that harbor carbapenemase of blaKPC-type. This agrees with other studies reporting that relebactam restored imipenem susceptibility in all K. pneumoniae with carbapenemases of blaKPC-2 and blaKPC-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 MBL 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 hydrolyze 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 Enterobacteriaceae carrying either bla_{amc} 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-lactamase. However, it showed no activity on MBL genes (bla_{NDM}, bla_{IMP}, bla_{VIM}) and efflux pump overactivity. Relebactam activity in IMP/NS K. pneumoniae with production of bla_{oxa-48} or with altered membrane permeability needs more study.

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