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Assessment of in vitro activity of ceftazidime/avibactam on carbapenemase-producing *Enterobacterales* from Iran: An experimental study

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Abstract

Background and Aims: The prevalence of carbapenemase-producing *Enterobacterales* (CPE) continues to increase worldwide. Combination of β-lactam and novel β-lactamase inhibitors introduce a revolutionary treatment option for CPE. Ceftazidime/avibactam (CAZ/AVB) has been recently developed for treatment of severe infections caused by multidrug-resistant bacteria. We aimed to evaluate in vitro activity of CAZ/AVB on a collection of 85 ESBL-producing-carbapenemase negative and CPE from Iran.

Methods: ESBL and carbapenemase production was phenotypically confirmed by combined disk test and modified carbapenem inactivation method respectively. The presence of clinically important carbapenemase encoding genes was examined using PCR. Susceptibility of all isolates to CAZ/AVB was determined using discs containing 30 µg ceftazidime +20 µg avibactam (AVB). Minimum inhibitory concentrations (MICs) of CAZ/AVB in 28 CPE (4 *Escherichia coli* and 24 *Klebsiella pneumoniae*) was determined by gradient diffusion method using MIC test strips (0.016–256 mg/L ceftazidime +4 mg/L AVB).

Results: All phenotypically identified ESBL positive-carbapenemase negative isolates were found to be susceptible to CAZ/AVB. Among the carbapenem resistant isolates, CAZ/AVB showed potent inhibitory activity against all OXA-48-like (MIC ranges 0.125/4–0.75/4 mg/L) and KPC positive isolates (MIC ranges <0.016/4–0.19/4 mg/L). However, AVB could not restore the activity of ceftazdime against isolates producing metallo-β-lactamases (MLBs) including VIM, NDM (MIC > 256/4 mg/L) and IMP (MIC > 8/4 mg/L).

Conclusion: Our data highlighted the excellent in vitro performance of CAZ/AVB against ESBL-producing and CPE suggesting that this combination can efficiently be used as therapeutic option for management of CPE infections particularly in regions with high prevalence of KPC and/or OXA-48-like positive but MBL-negative *Enterobacterales*.

KEYWORDS

carbapenem resistant *Enterobacterales*, ceftazidime- avibactam, ESBL, β-lactamase inhibitor

1 | INTRODUCTION

Antibiotic resistance among bacteria has reached to an alarming level necessitating availability of novel agents, particularly those which can overwhelm the pre-existing resistance mechanisms. β-lactams remain the most widely used class of antibiotics in clinical practice.[1] Carbapenems, are known as the *most* effective group of *β-lactam* (BL) family of antibiotics, with a broad spectrum of antimicrobial activity and the inherent stability against a variety of β-lactamases. They are often used as last-resort therapeutic options, for treating infections caused by multidrug-resistant (MDR) bacteria.[2,3] However, the efficacy of these antimicrobials is hampered by emergence of carbapenem hydrolyzing enzymes produced by major Gram negative pathogens particularly *Enterobacterales*. Carbapenemase producing *Enterobacterales* (CPE) are ranked as one of the most urgent priorities among antibiotic-resistant pathogens for research and development of new antibiotics by World Health Organization.[4] Carbapenem resistance among CPE is attributed to production of Ambler Class A [klebsiella pneumoniae carbapenemase (KPC)], Class B metallo-β-lactamases (MBLs) [New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP)] and class D [oxacillinase-48 (OXA-48)-like] carbapenemases. The distribution of carbapenemase producers varies worldwide. While Middle East (including Turkey and Iran) and North African countries are considered the principal reservoirs of the OXA-48 producers,[5] the KPC carbapenemases have shown high frequencies in North[6] and Central America and also some European countries (Greece, Italy).[7] On the other hand Asian continent (mostly India and China) serves as the major reservoir of NDM producers.[7] NDM, and OXA-48-like carbapenemases are reported to be the most predominant enzymes among clinical CPE from Iran.[8,9]

Since CPE often exhibit non-susceptibility to a broad range of antibiotic classes, treatment of CPE infections is extremely challenging and commonly includes prescription of polymyxins and tigecycline for which elevated rate of resistance has been increasingly reported.[10] An effective strategy to restore the effectiveness of β-lactam antibiotics is pairing a β-lactam with β-lactamase inhibitor (BLI). Clavulanic acid, sulbactam and tazobactam, were the first BLIs which were co-formulated with β-lactams such as amoxicillin, ampicillin and piperacillin respectively in a global effort to prevent β-lactam resistance. However, these old BLIs were active only against Gram-negative bacilli harboring Ambler class A enzyme, including some isolates of extended-spectrum beta-lactamase (ESBL) producing *Enterobacterales*.[11] Recently, novel BLI combinations have been introduced providing new therapeutic options for CPE infections. Avibactam (AVB), which belongs to diazabicyclooctane group of BLIs was approved for use in combination with the third generation cephalosporin ceftazidime for intravenous therapy of complicated urinary tract infections, complicated intra-abdominal infections and hospital-acquired pneumonia/ventilator-associated pneumonia, caused by MDR Gram-negative bacterial pathogens.[12,13] AVB has been shown to inhibit a broad spectrum of β-lactamases, including Ambler Classes A (sulfhydryl reagent variable (SHV), cefotaximase (CTX-M), and KPC) and C (AmpC), as well as some class D (OXA-48), with high affinity. However, it does not improve the activity of ceftazidime against class B MBLs (IMP, VIM, and NDM).[12,14] Several reports have been published on the in vitro activity of CAZ/AVB against CPE.[15,16] However, limited data evaluating the activity of this BL/BLI combination on CPE are available from Iran. Therefore, we aimed to evaluate the in vitro activity of CAZ/AVB against serine and MLB producing *Enterobacterales* isolated from different clinical samples in Iran.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates

About 85 clinical ESBL-producing-carbapenemase negative and carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates obtained from inpatients and outpatients in three different hospitals were included in this study. The bacterial isolates were selected from our microbial collection which were obtained during the past 2 years and preserved at −80° C. The studied bacterial isolates were identified by using conventional biochemical methods[17] and only imipenem (for carbapenemase production) or ceftazidime resistant bacteria (for ESBL production) were included and all ceftazidime susceptible bacteria were excluded from the study. In the case of CPE isolates we tried to include all 5 clinically important carbapenemase (NDM, VIM, IMP, OXA-48-like, and KPC)-expressing isolates to test the effect of AVB on each type of class A, B, and D type carbapenemases. Therefore, the included samples did not reflect the real prevalence of carbapenemase enzymes in this study. Ethical approval was waived by the local Ethics Committee of University of Tabriz as all isolates in this study were granted from the bacterial collection of hospital for research purposes and no direct human samples were used.

2.2 | Phenotypic confirmation of ESBL and carbapenemase production

ESBL production was phenotypically confirmed by the combined disk test (CDT) method using ceftazidime ([30 μg]) discs alone and ceftazidime-clavulanate (CAZ/CL) (30 + 10 μg) discs based on CLSI recommendations. ESBL-producing strains were recognized when a difference of at least 5 mm in the inhibition zone diameter of CAZ/CL versus CAZ was obtained. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as control strains. Testing susceptibility to imipenem was performed by broth dilution method using antibiotic powder from Glentham Life sciences (UK). Phenotypic detection of carbapenemase production was performed using modified carbapenem inactivation method (mCIM) as described by CLSI. Briefly, 2 mL of trypticase soy broth was inoculated with loopful of tested bacteria, and a 10 μg meropenem disk was placed into the mixture. Following incubation for 4 h (±15 min) the meropenem disk was removed from

the tube and placed on a Mueller-Hinton agar plate seeded with a 0.5Mc Farland suspension of *E. coli* ATCC 25922. mCIM was interpreted as positive (detection of carbapeneamse) when the inhibition zone diameter was 6–15 mm, or 16–18 mm with small colonies in the inhibitory zone.[18]

2.3 | DNA extraction and detection of carbapenemase encoding genes

Total DNA was extracted using the boiling method. Briefly, a loop full of bacteria from a plate was picked and transferred to the Eppendorf tube containing 200 µL TE buffer. The suspension was boiled for 5–10 min, centrifuged for 5 min at 12,000*g*, and the obtained supernatant was used as DNA template. Carbapenemase genes including bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{OXA\text{-}48\text{-}like}$ were amplified using the gene specific primers.[19] PCR reactions were performed using Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Co) according to manufacturer's protocol. PCR products were analyzed by agarose gel electrophoresis.

2.4 | Testing susceptibility to ceftazidime/AVB (CAZ/AVB) by disc diffusion and gradient diffusion methods

The susceptibility of all 85 bacterial isolates (including carbapenem susceptible and resistant) to CAZ/AVB was tested by disc diffusion method using disc containing 30 µg ceftazidime and 20 µg AVB (Mast Group Ltd, UK, lot 466159). Moreover, the minimum inhibitory concertation (MIC) of CAZ-AVB in 28 CPE (4 *E. coli* and 24 *K. pneumoniae*) was determined using MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy, lot 060821030) containing concertation gradient range of 0.016/4–256/4 mg/L. The MIC was read directly from the scale at the point where the growth inhibition ellipse intersected the MIC test strip. The obtained results were interpreted according to CLSI M100-Ed32 guidelines (MIC breakpoints: resistant, >8/4 mg/L; Zone diameter breakpoint: Resistant, ≤20 mm).

Inter-method agreement between disc diffusion and gradient diffusion methods and also association between presence of specific resistance gene and susceptibility to CAZ/AVB was assessed using kappa test and Spearman's correlation (two-tailed test) respectively and $p < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics (V26).

3 | RESULTS

A total of 85 bacterial isolates of *K. pneumoniae* ($n = 38$) and *E. coli* ($n = 47$) were included in this work. While all *E. coli* isolates included in this study were obtained from urine, *K. pneumoniae* isolates had been obtained from urine, sputum, wound and blood samples. The activity of CAZ/AVB was tested against ESBL-positive, carbapenemase-negative *Enterobacterales*, ($n = 57$) and carbapenemase producers ($n = 28$, 4 *E. coli*, 24 *K. pneumoniae*) using disc diffusion. The MIC test strip was used for MIC determination of CAZ/AVB in the latter group of tested bacteria. All studied CPE isolates were characterized with positive mCIM results (Figure 1) and imipenem MICs ≥ 4 mg/L and carried OXA-48-like ($n = 12$), NDM ($n = 3$), NDM + OXA-48like ($n = 5$), KPC ($n = 3$), VIM ($n = 4$) and IMP ($n = 1$). Table 1 displays the imipenem MIC distribution in carbapenemase-producing isolates.

All phenotypically identified ESBL positive-carbapenemase negative isolates were found to be susceptible to AVB as determined by disc diffusion. Among the carbapenem resistant isolates, CAZ/AVB showed potent inhibitory activity against all serine carbapenemase, OXA-48-like and KPC positive isolates. The MIC ranges of CAZ-AVB against OXA-48-like and KPC producing bacteria were found to be 0.125/4–0.75/4 mg/L and <0.016/4–0.19/4 mg/L respectively. On the other hand, AVB couldn't restore the activity of ceftazdime against isolates producing MBLs including VIM, NDM, and IMP. Indeed, all NDM and VIM producing isolates revealed high level of resistance to CAZ/AVB being characterized with MICs > 256/4 mg/L (strong association with resistance according to Spearman's correlation test, $p < 0.001$) (Figure 2 and Table 2).

Comparison of CAZ/AVB susceptibility testing results obtained by gradient diffusion and disc diffusion showed a high level of agreement producing a categorical agreement rate of 100% between the two methodologies (kappa = 1.00, $p < 0.001$) with no very major error (false-susceptible result) or major error (false-resistant result). The inhibition zone diameters of CAZ/AVB against phenotypically confirmed ESBL-positive isolates were found to be 24–33 mm and for KPC positive isolates ranged from 22 to 23 mm, OXA-48-like isolates, 21–26 mm and VIM, NDM and IMP positive isolates, 11–14 mm, 7–16 mm and 12 mm respectively.

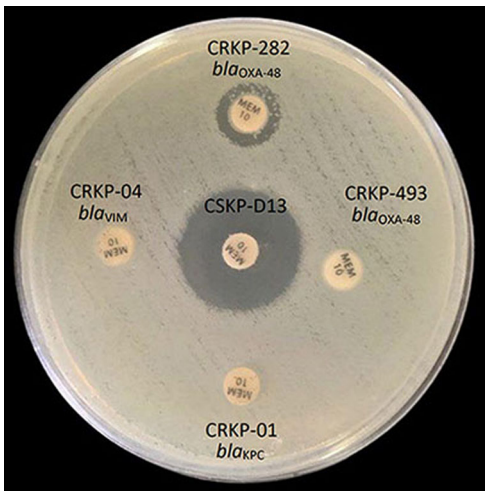

FIGURE 1 Results of modified carbapenem inactivation method (mCIM) for carbapenemase production. CRKP, carbapenem resistant *Klebsiella pneumoniae*; CSKP, carbapenem susceptible *K. pneumoniae*.

4 | DISCUSSION

Ceftazidime-AVB is a combination of novel BLI and ceftazidime which expands ceftazidime's spectrum of activity to include many ceftazidime- and carbapenem-nonsusceptible *Enterobacterales* by actively inhibiting Ambler class A, class C and some class D β-lactamases.[20] In the current work we evaluated the in vitro activity of CAZ/AVB against ESBL and carbapenemase positive isolates. According to disc diffusion results, CAZ/AVB revealed potent activity against phenotypically confirmed ESBL-positive isolates, including 14 *K. pneumoniae* and 43 *E. coli*, isolates. These data are consistent with reports from other surveillance studies on clinical ESBL-producing *Enterobacterales*.[21,22] On the other hand, the activity of CAZ-AVB on CPE was dependent on the type carbapenemase harbored by the pathogen. In this study only CPE harboring OXA-48-like and KPC enzymes were highly susceptible to CAZ-AVB (MICs ≤ 0.75/4 and ≤0.19/4 mg/L respectively). However, AVB was not active on IMP, VIM and NDM-positive isolates. Infections caused by OXA-48-like producing *K. pneumoniae* in Iran is rising and several studies from Iran have reported this class D serine β-lactamase enzyme as the most frequent carbapenemase detected among CPE.[23,24] Since its first description in turkey in 2001, this type of class D β-lactamase has rapidly spread globally and it is considered as an endemic and the most prevalent enterobacterial carbapenemase across middle east [25] introducing CAZ/AVB as a potentially efficient treatment option for infections caused by OXA-48-like producing bacteria in this region. In addition to OXA-48-like enzyme, CAZ/AVB

TABLE 1 Distribution of imipenem MICs against carbapenemase producing *Escherichia coli* and *Klebsiella pneumoniae*.

Bacteria with carbapenemase	No. of isolates inhibited at respective MIC (mg/L)				
	4	8	16	32	≥64
KPC (*n* = 3)			2	1	
OXA-48-like (*n* = 12)	10	1		1	
NDM (*n* = 3)					3
NDM + OXA-48-like (*n* = 5)				1	4
VIM (*n* = 4)					4
IMP (*n* = 1)			1		

Abbreviation: MICs, minimum inhibitory concentrations.

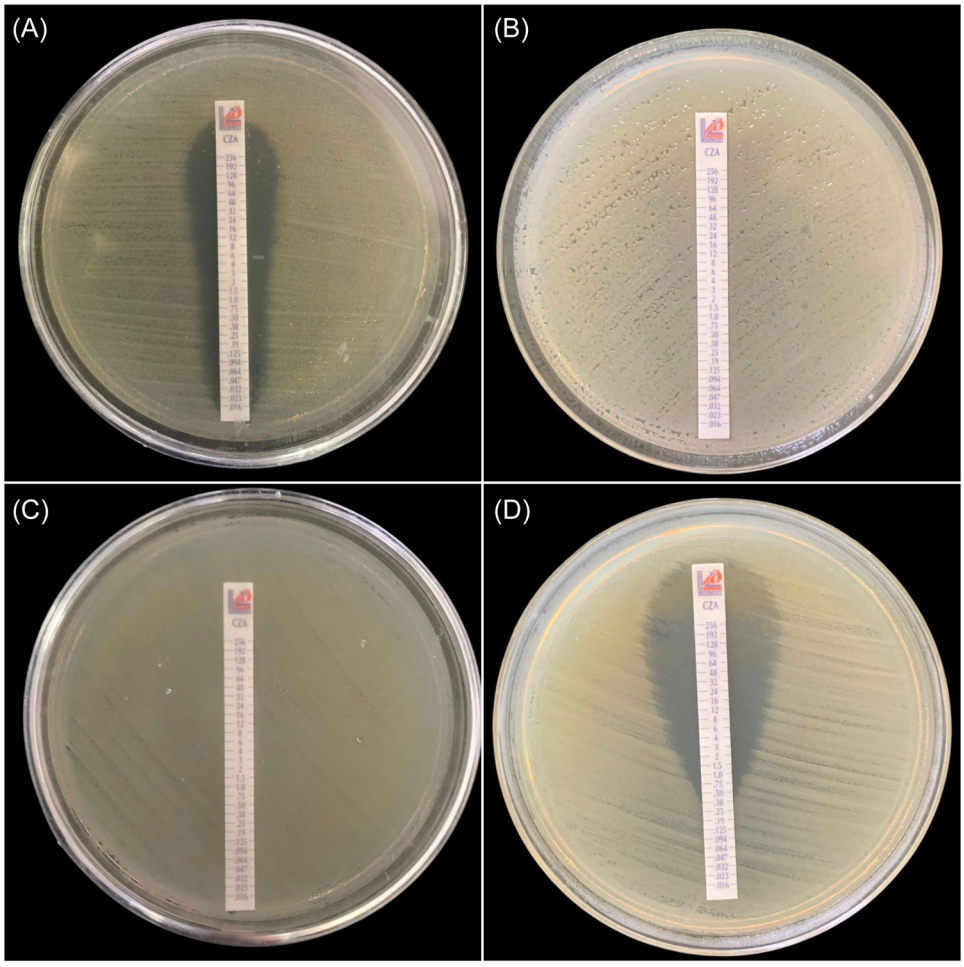

FIGURE 2 Susceptibility testing with ceftazidime/avibactam MIC test strips against carbapenemase producing *Enterobacterales*. (A) KPC positive; (B) VIM positive; (C) NDM + OXA-48-like positive; (D) OXA-48-like positive isolate. MICs, minimum inhibitory concentrations.

TABLE 2 The MIC values of ceftazidime-avibactam combination tested on carbapenemase producing isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

	Number (%) of isolates with MICs (mg/L)										
Bacteria	**≤0.016–0.023**	**0.032–0.047**	**0.064–0.094**	**0.125–0.19**	**0.25–0.38**	**0.5–0.75**	**1–8**	**12–16**	**2–96**	**128–192**	**≥256**
K. pneumoniae (*n* = 24)											
OXA-48-like (*n* = 12)				**2 (7.1)**	**3 (10.7)**	**7 (25)**					
NDM + OXA-48-like (*n* = 4)											**4 (14.2)**
VIM (*n* = 4)											**4 (14.2)**
IMP (*n* = 1)								**1 (3.5)**			
KPC (*n* = 3)	**1 (3.5)**			**2 (7.1)**							
Escherichia coli (*n* = 4)											
NDM (*n* = 3)											**3 (10.7)**
NDM + OXA-48like (*n* = 1)											**1 (3.5)**

Abbreviation: MICs, minimum inhibitory concentrations.

showed a potent activity against KPC producers. While, KPC carbapenemases are constituting the major carbapenemase in North America,[6] Europe (Greece[26] and Italy[27]), and some regions of China, it is not common in Iran and several studies have reported low prevalence or even lack of this enzyme among studied *K. pneumoniae* isolates form Iran.[23,24] The CAZ/AVB has been reported to have excellent in vitro activity against ESBL-producing *Enterobacterales*, CPE and KPC-producing *Enterobacterale* in other studies.[16,28] Moreover, CAZ/AVB has been clinically proven to be effective in treatment of neonatal bacteremia caused by ESBL or KPC-positive *K. pneumoniae* in a study from Italy.[29] Significantly improved clinical success and survival was reported for patients with KPC or OXA-48 carbapenemase producing *Enterobacteriaceae* who received CAZ-AVB in studies from different geographic regions.[30,31] CAZ/AVB treatment outcomes in infections caused by CPE has been reported to rely on infection type so that pneumonia and mechanical ventilation have been found to increase the risk of treatment failure.[13] Despite good clinical outcomes of CAZ/AVB against OXA-48 and KPC producers, developing CAZ/AVB resistance due to previously reported KPC mutations (KPC-2 D179Y) could be a rising global issue in the treatment of CPE infection.[13,32] As it has been previously demonstrated, AVB could not inhibit any of the MLBs, IMP, VIM and NDM in this study. This is in accordance with findings of a study from India (where NDM-expressing isolates are common) in which 51% and 24% of carbapenem resistant *K. pneumoniae* and *E. coli* isolates were found to be susceptible to CAZ-AVB respectively.[15] The NDM is the most frequent MBL identified among *Enetrobacterlase* from Iran and coproduction of NDM with OXA-48-like is increasingly being reported form this country.[23] So far there is no study assessing the clinical outcomes of CPE infection treatment with CAZ/AVB containing regimens from Iran. It is predicted that AVB can be used in combination with CAZ as a good therapeutic option for CPE infections in this country where OXA-48-like carbapenemases are the most common enzymes. However, increasing coproduction of NDM along with this class D carbapenemase raises the concern about the limited effectiveness of this BL/BLI combination on Iranian CPE in the future. Aztreonam-AVB is another BL/BLI combination which shows potent activity against CAZ/AVB resistant CPE due to stability of aztreonam against MBLs paired with anti- Class A, Class C, and (some) Class D serine-β-lactamase activity provided by AVB.[33] In addition to diazabicyclooctane-derived compounds such as avibavtam and relebactam which are not able to inhibit MBLs, the boronic acid derivatives, such as taniborbactam and Xeruborbactam (QPX7728) display anti serine-β-lactamase and MBL activity and offer additional therapeutic alternatives against CPE including MLB producing isolates.[34–36]

5 | CONCLUSION

In conclusion, the results of our study indicated that ceftazidime/AVB can overcome resistance attributed to ESBLs, and carbapenemases with serine active sites (e.g., KPC and oXA-48-like), but does not inhibit the growth of isolates producing MLBs. Therefore, CAZ/AVB can be considered as an excellent antimicrobial agent against KPC and/or OXA-48-like positive but MBL-negative isolates. As OXA-48-like is the most common carbapenemase harbored by Iranian CPE, CAZ/AVB provides promising therapeutic option for treatment of CPE infections in Iran. However, increasing prevalence of NDM, an AVB-resistant enzyme among Iranian isolates, may limit the efficacy of this BL/BLI combination for treatment of CPE infection in the future necessitating availability of other BLIs with anti-MBL activity

(such as taniborbactam or Xeruborbactam) for management of infections caused by these difficult-to -treat pathogens.

AUTHOR CONTRIBUTIONS

Mehri Haeili: Conceptualization; investigation; writing—review and editing; project administration; supervision; resources; **Parisa Ghaderi Bavil-Olyaei**: Methodology; validation; investigation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

All authors have read and approved the final version of the manuscript and CORRESPONDING AUTHOR has full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

TRANSPARENCY STATEMENT

The lead author Mehri Haeili affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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