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Empfindlichkeit und molekulare Resistenzmechanismen gegenüber Ceftazidim/Avibactam in gramnegativen Mikroorganismen aus fünf lateinamerikanischen Ländern

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Zusammenfassung

Resistenzen gegenüber Carbapenemen sind eine Bedrohung für die globale Gesundheit mit wenigen verbleibenden Therapieoptionen.
Ceftazidim/Avibavtam (CZA) ist die Kombination aus einem Cephalosporin und einem Diazabicyclooctan, mit der Eigenschaft eine Vielzahl von Carbapenemasen der Ambler Klasse A und D zu inhibieren. Resistenzen gegenüber Carbapenemen in gramnegativen Bakterien sind in Kolumbien und anderen Ländern Lateinamerikas weit verbreitet. In den hier vorgestellten Arbeiten wurden 2.235 Enterobakterien und 492 *P. aeruginosa* Isolate aus fünf Lateinamerikanischen Ländern auf ihre Empfindlichkeit gegenüber CZA und anderen klinisch verfügbaren Antibiotika untersucht. Die CZA-resistenten Isolate wurden mittels PCR und Genomsequenzierung auf die zugrundeliegenden Resistenzmechanismen hin analysiert. CZA zeigte Aktivitäten gegenüber 99,2% (2.217/2.235) aller untersuchten Enterobacterales und 77,8% (383/492) aller *P. aeruginosa* Isolate. Als plausible Erklärung für die Resistenz gegen CZA konnte mittels qPCR bei allen Enterobakterien und bei 38,5% (42/109) der *P. aeruginosa* Isolate ein Metallo-β-Laktamase (MBL)-kodierendes Gen nachgewiesen werden. Die verbleibenden *P. aeruginosa* Isolate wurden einer Genomsequenzierung unterzogen, dabei zeigten sich Mutationen in Genen, die zuvor mit einer verringerten Empfindlichkeit gegen CZA assoziiert wurden, wie z.B. Genen die mit der Überexpression von MexAB-OprM und AmpC (PDC) in Verbindung stehen, sowie Genen, die PoxB (*bla*$_{\text{OXA-50-like}}$), FtsI (PBP3), DacB (PBP4) und OprD kodieren. Unsere Ergebnisse unterstreichen die Notwendigkeit von Therapieoptionen gegenüber MBL-produzierenden und anderen Carbapenem-resistenten Mikroorganismen. Des Weiteren sind diese Studien eine Momentaufnahme der Empfindlichkeit gegen CZA vor dessen Verfügbarkeit in Lateinamerika und dienen deswegen als Ausgangspunkt um die Entwicklung von Resistenzen in dieser Region zu verfolgen.

Summery

Resistance to carbapenems is a global public health threat with only few remaining therapeutic options. Ceftazidime/avibavtam (CZA) is the combination of a cephalosporin and a diazabicyclooctane that inhibits a variety of Ambler class A and D carbapenemases. In Colombia and other Latin American countries, high rates of carbapenem resistance among Gram-negative bacilli has been reported. In the studies presented here, 2.235 *Enterobacterales* and 492 *P. aeruginosa* isolates from five Latin American countries were tested for their susceptibility to CZA and other clinically available antibiotics. Furthermore, the CZA-resistant isolates were analyzed for the underlying resistance mechanisms using qPCR and whole genome sequencing. CZA showed activity against 99,2% (2.217/2.235) of all *Enterobacterales* and 77,8% (383/492) of *P. aeruginosa* isolates tested. As a plausible explanation for the resistance to CZA, a metallo-β-lactamase (MBL)-coding gene could be detected by qPCR in all *Enterobacterales* and in 38,5% (42/109) of the *P. aeruginosa* isolates. The remaining *P. aeruginosa* isolates were subjected to whole genome sequencing. The analysis revealed mutations in genes previously associated with decreased susceptibility to CZA, such as genes associated with overexpression of MexAB-OprM and AmpC (PDC), as well as genes encoding PoxB (*bla*$_{\text{OXA-50-like}}$), FtsI (PBP3), DacB (PBP4) and OprD. Our results highlight the need for therapeutic options against MBL-producing and other carbapenem-resistant microorganisms. In addition, these studies provide a snapshot of susceptibility to CZA prior to its availability in Latin America and therefore serve as a starting point to trace the evolution of resistance in this region.

Übergreifende Zusammenfassung

Resistenz gegenüber β-Laktamen

Antibiotikaresistenzen sind eine Bedrohung für die globale Gesundheit. Nach aktuellen Schätzungen verstarben im Jahr 2019 insgesamt 1,27 Million Menschen direkt an den Folgen von Antibiotikaresistenzen und weitere 3,68 Millionen Todesfälle standen damit in Zusammenhang (1). Mikroorganismen wie Methicillin-resistente *Staphylococcus aureus*, Vancomycin-resistente Enterokokken, Breitbandspektrum β-Laktamasen- (extended-spectrum β-lactamases; ESBL) und Carbapenemasen-produzierende Enterobakterien, sowie Carbapenem-resistente *Pseudomonas*- und *Acinetobacter*-Stämme sind weltweit für die Mehrheit der Todesfälle verantwortlich (2). Während sich Antibiotika-resistente Mikroorganismen durch den Gebrauch von Antiinfektiva in der Landwirtschaft, Veterinär- und Humanmedizin verbreiten, befinden sich nur wenige neue Substanzen in Entwicklung (2,3).

β-Laktame bilden den Grundstein der empirischen, wie auch der gezielten antibakteriellen Therapie, weswegen Resistenzen gegenüber dieser Klasse besonders problematisch sind. Resistenzen gegenüber β-Laktamen werden durch i) Expression von Enzymen, den sogenannten β-Laktamasen, ii) Änderungen der Permeabilität der bakteriellen Zellmembran, iii) Expression von Effluxkanälen, iv) Mutationen, die zu Änderungen der Affinität des Antibiotikums gegenüber dem pharmakologischen Ziel führen oder einer Kombination mehrerer dieser Mechanismen hervorgerufen. In Enterobakterien ist die Produktion von β-Laktamasen, die häufigste Ursache für ein eine Resistenz gegenüber dieser Klasse, während bei *P. aeruginosa* häufig mehrere Mechanismen zusammenspielen (4).

Carbapenemasen sind Enzyme mit der Fähigkeit nahezu alle verfügbaren β-Laktame zu hydrolysieren und somit unwirksam zu machen (5). Zur Therapie von Patienten mit Infektionen durch Carbapenem-resistente Mikroorganismen verblieben bis vor kurzem nur wenige, meist nebenwirkungsreichere oder weniger effektive Optionen wie Polymyxine, Amikacin, Tigecyclin und

Fosfomycin (5,6). Seit einigen Jahren sind Kombinationen aus β-Laktam und nicht β-Laktam-basierenden β-Laktamase-Inhibitoren in der Klinik verfügbar. Ceftazidim/Avibactam (CZA) ist die Kombination aus einem Cephalosporin der dritten Generation und einem neuen Diazabicyclooctan-β-Laktamase-Inhibitor mit der Fähigkeit verschiedene β-Laktamasen der Ambler Klasse A, C und D reversibel zu hemmen, inklusive der weltweit verbreiteten Carbapenemasen der KPC-Familie (7).

Mikroorganismen können basierend auf der Expression einer Metallo-β-Laktamase (MBL) (Ambler Klasse B) oder aufgrund von nicht enzymatischen Mechanismen eine Resistenz gegenüber Carbapenemen entwickeln. Diese Bakterien sind in der Regel gegen die klinisch verfügbaren Kombinationen aus β-Laktam/β-Laktamase-Inhibitoren resistent. Aus diesem Grund ist die Charakterisierung der zirkulierenden Mechanismen, die zur Resistenz gegenüber Carbapenemen führen von großer Bedeutung.

In Kolumbien und anderen Ländern Lateinamerikas wurde in der Vergangenheit eine hohe Prävalenz von multiresistenten gramnegativen Mikroorganismen beschrieben (8). In einem Review aus dem Jahr 2021 haben wir die veröffentlichten Beschreibungen von Carbapenemasen in Lateinamerika zusammengefasst (9). Neben den weitverbreitenten Enzymen der Familien KPC, NDM, VIM und IMP wurden zahlreiche weitere Carbapenemasen beschreiben. KPC-Enzyme sind in einigen Ländern Lateinamerikas endemisch und werden zunehmend in Mikroorganismen wie *Raoultella* spp., *Serratia* spp. und *Morganella* spp. beschrieben (9,10). Ebenso häufen sich die Berichte über den Nachweis von MBL (insbesondere NDM und IMP) in Enterobakterien, sowie die Expression von zwei oder mehr Carbapenemasen (11).

Wie bereits dargelegt, ist die Resistenz gegenüber Carbapenemen, die durch vielfältige Mechanismen hervorgerufen werden kann, problematisch, da häufig nur wenige Therapieoptionen verbleiben. In Lateinamerika wurde eine hohe Rate an Carbapenem-resistenten Mikroorganismen, sowie eine Vielzahl von zirkulierenden Carbapenemasen beschrieben. Ziel der vorliegenden Arbeiten war es, die Empfindlichkeit gegenüber Ceftazidim/Avibactam und die zugrundeliegenden Resistenzmechanismen zu eruieren.

Aktivität von CZA gegenüber Enterobacterales aus Lateinamerika

In der Arbeit „In vitro susceptibility to ceftazidime/avibactam and comparators in clinical isolates of *Enterobacterales* from five Latin American countries", wurde die Aktivität von CZA und anderen klinisch verfügbaren Antibiotika untersucht (12). Wir analysierten 2.252 *Enterobacterales*, die zwischen Januar 2016 und Oktober 2017 aus klinischen Proben aus verschiedenen Zentren in Argentinien, Brasilien, Chile, Kolumbien und Mexiko isoliert wurden.

Die antimikrobielle Empfindlichkeitstestung wurde mittels Mikrodilution durchgeführt und sofern verfügbar, nach den Kriterien des Clinical and Laboratory Standards Institute (CLSI) von 2018 interpretiert (13). Zur Interpretation der Aktivität von Fosfomycin wurden die für *E. coli* verfügbaren CLSI-Kriterien von 2018 angewendet (Fosfomycin empfindlich <128 mg/l) (13), während für die Interpretation von Tigecyclin, die Kriterien der United States Food and Drug Administration angewendet wurden (empfindlich: ≤2 mg/l, intermediär: 4 mg/l; resistent ≥8 mg/l) (14).

Insgesamt war CZA gegenüber 95,8% (2.157/2.252) aller *Enterobacterales* aktiv: Die höchste Aktivität zeigte CZA gegenüber *E. coli* (97,9%; 1.379/1.409), während die geringste gegenüber *E. cloacae* complex Isolaten (92%; 103/112) beobachtet werden konnte. Mehr als 90% aller untersuchten Enterobakterien wiesen eine minimale Hemmkonzentration (minimum inhibitory concentration; MIC) ≤1 mg/l auf. Neben CZA waren Fosfomycin und Tigecyclin die Substanzen mit der höchsten Aktivität (beide 93,4%; 2.103/2.252), gefolgt von Meropenem (88,7%; 1.998/2.252) und Imipenem (87,1%; 1.961/2.252), während Ceftazidim (64%; 1.441/2.252) die geringste Aktivität aufwies. Die höchste Empfindlichkeit gegenüber CZA wurde unter den Isolaten aus Chile beobachtet (94,8%; 420/443), während die der kolumbianischen Isolate am niedrigsten war (71,3%; 995/1.396).

Zweihundertachtundsechzig (17,6%) der untersuchten Isolate waren nicht gegenüber Ertapenem empfindlich, d.h. sie wiesen eine MIC von Ertapenem ≥1 mg/l auf, entsprechend der CLSI-Kategorie intermediär. Innerhalb dieser Subgruppe der nicht gegenüber Carbapenem empfindlichen Isolate (carbapenem non-susceptible; CNS), waren 77,5% (307/396) gegenüber CZA

empfindlich. Fosfomycin war gegen 81,3% (322/396) und Tigecyclin gegen 68,9% (273/396) der Isolate aktiv. Meropenem zeigte gegenüber 35,9% (154/396) der CNS-Isolate Aktivität. Die höchste Aktivität zeigte CZA gegenüber den CNS-Isolaten aus Chile (94,8%; 55/58), gefolgt von Mexico (93,3%; 13/15), Brasilien (88,6%; 31/35), Argentinien (80%; 16/20) und Kolumbien (71,3%; 191/268). Die meisten Isolate der CNS-Gruppe waren *K. pneumoniae* (46,2%; 183/396), gefolgt von *E. coli* (34,6%; 137/396), die Mehrheit (67,7%; 268/396) wurde in kolumbianischen Gesundheitseinrichtungen isoliert.

Obwohl insgesamt eine hohe in vitro Aktivität von CZA gegenüber klinischen Isolaten aus fünf lateinamerikanischen Ländern beobachtet werden konnte, sind Resistenzen gegenüber dieser Substanz besorgniserregend – zum einen da CZA zum Zeitpunkt der Studie nicht in Lateinamerika verfügbar war und zum anderen, da in der Klinik kaum therapeutische Optionen verbleiben. Die relativ hohe Rate an CZA-Resistenz unter den untersuchten CNS-Isolaten, ist am ehesten durch die Expression von MBL und/oder dem Vorliegen von nicht-enzymatischen Mechanismen allein oder in Kombination mit der Expression von Serin-β-Laktamasen zu erklären.

Carbapenemasen-kodierende Gene in CZA-resistenten gramnegativen Bakterien aus Kolumbien

Weitere Untersuchungen stützen die Hypothese der Expression von MBL: Die kolumbianischen, CZA-resistenten Isolate wurden mittels PCR auf fünf der häufigsten Carbapenemase-kodierende Gene (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$ und *bla*$_{OXA-48}$) untersucht (15). In 18 der 79 (22,8%) Isolate konnte ein MBL-kodierendes Gen allein oder zusammen mit *bla*$_{KPC}$ nachgewiesen werden. In 61 Isolaten (77,2%) konnte keines der fünf weltweit relevantesten Carbapenemase-kodierenden Gene gefunden werden konnte.

Neben den erwähnten Enterobakterien wurden 131 CNS *P. aeruginosa* Isolate (*P. aeruginosa* mit MIC für Meropenem ≥4 mg/l) aus 13 verschiedenen kolumbianischen Gesundheitseinrichtungen analysiert: 45% (59/131) der Isolate waren empfindlich gegenüber CZA, gefolgt von Piperacillin/Tazobactam (30%; 39/131), Cefepim (29%; 38/131) und Ceftazidim (27%; 35/131). Unter den 70

untersuchten CZA-resistenten *P. aeruginosa* Isolaten konnte *bla*$_{VIM}$ in 22 (31,4%), *bla*$_{KPC}$ in 20 (28,6%) und die Kombination aus beiden in 6 (8,6%) Isolaten nachgewiesen werden. In weiteren 22 Isolaten (31,4%) konnte keines der untersuchten Carbapenemase-kodierenden Gene nachgewiesen werden.

Molekulare Resistenzmechanismen in CZA-resistenten *Enterobacterales* und *P. aeruginosa* Isolaten

In der Arbeit „Molecular mechanisms of resistance to ceftazidime/avibactam in clinical isolates of *Enterobacterales* and *Pseudomonas aeruginosa* in Latin American hospitals“ untersuchten wir 492 *P. aeruginosa* Isolate, sowie 2.235 der zuvor beschriebenen *Enterobacterales* Isolate. Nach dem Umzug des Labors von Cali nach Bogotá im Jahr 2018 konnten 17 Stämme (zwölf *E. coli*, drei *K. pneumoniae* und jeweils ein Isolat von *K. aerogenes* und *S. marcescens*) nicht aufgefunden werden und deswegen nicht weiter analysiert werden. Ziel der Arbeit war es, die molekularen Mechanismen, die zur Resistenz gegenüber CZA führen zu beschreiben.

Nach Überprüfung der Empfindlichkeit gegenüber CZA mittels Mikrodilution (Sensititer® plates, Trek Diagnostic Systems, Thermo Fisher, UK) und E-Test (bioMérieux, Marcy-l'Étoile, France), konnte lediglich bei 0,8% (18/2.235) aller untersuchten Enterobakterien eine Resistenz bestätigt werden. Alle 18 Isolate stammten aus klinischen Proben aus neun verschiedenen kolumbianischen Zentren. Anschließend wurden die Stämme mittels RAPIDEC® Carba-NP Assay (bioMérieux, Marcy-l'Étoile, France), einem phänotypischen Test, bei welchem ein positives Ergebnis auf die Expression einer Carbapenemase hindeutet, untersucht. Bei allen Isolaten fiel der Test positiv aus. Mittels qPCR konnte in allen Isolaten ein MBL-kodierendes Gen nachgewiesen werden: bei 17 Isolaten *bla*$_{NDM}$ und in einem *bla*$_{VIM}$ nachgewiesen werden. Vier der Isolate wären zusätzlich Träger von *bla*$_{KPC}$. Da die Resistenz gegenüber CZA durch MBL-Expression plausibel erklärt werden kann, wurde auf eine Sequenzierung der Isolate verzichtet.

Von den 492 untersuchten *P. aeruginosa* Isolaten waren 109 (22,2%) CZA-resistent. Ebenso konnte mittels qPCR in 42 Isolaten (38,9%) ein MBL-

kodierendes Gen nachgewiesen werden: 38 der 109 Isolate (34,9%) beherbergten *bla*$_{VIM}$, sieben davon in Kombination mit *bla*$_{KPC}$, drei Isolate *bla*$_{IMP}$ und eins *bla*$_{SPM-1}$. Die verbleibenden 67 Isolate (61,5%) bei welchen kein MBL-kodierendes Gen als mögliche Erklärung für eine Resistenz gegenüber CZA vorgefunden werden konnte, wurden mittels Illumina MiSeq platform (Illumina Inc., San Diego, California, USA) sequenziert. Pro sequenziertem Isolat erhielten wir zwischen 110 und 137 Contigs mit einer Länge der Assemblierungen zwischen 6,3 und 7,2 Kb. Der GC-Anteil lag zwischen 65,8% und 66,5%. Sechs Isolate wurden aufgrund einer unerwartet niedrigen Sequenzabdeckung (<30× pro Base) von der anschließenden Analyse ausgeschlossen: Bei fünf dieser Isolate konnte mittels qPCR keine Carbapenemase nachgewiesen werden, eines war Träger von *bla*$_{KPC}$.

Insgesamt konnten 23 bekannte und fünf bisher unbekannte Sequenztypen (ST) beschrieben werden. Am häufigsten wurde der ST235 (16/61; 26,2%) identifiziert, gefolgt von ST575 (9/61; 14,8%) und ST309 (6/61; 9,8%). Alle untersuchten Isolate beherbergten die beiden Gene *bla*$_{AmpC}$ und *bla*$_{OXA-50-like}$. In 17 Isolaten (27,9%) konnte *bla*$_{OXA-2}$ nachgewiesen werden, elf davon gehörten zum ST235 und vier zum ST309. Das Carbapenemase-kodierende Gen *bla*$_{GES-19}$ wurde in vier Isolaten (6,6%) aus Mexiko beobachtet; drei davon wurden dem ST235 zugeordnet. Ein weiteres Isolat (1,6%) des ST309 beherbergte *bla*$_{GES-19}$ und *bla*$_{GES-20}$ in einer Tandemkonfiguration. In jeweils einem Isolat aus Argentinien und Chile konnte respektiv *bla*$_{PER-1}$ und *bla*$_{PER-3}$ beschrieben werden.

Sieben *P. aeruginosa* Isolate (11,5%) des ST235 waren Träger von *bla*$_{KPC-2}$, jedoch konnte bei keinem Isolat eine Mutation in diesem Gen nachgewiesen werden, die eine Resistenz gegenüber CZA erklären könnte. Diese Isolate wurden daher auf weitere Mutationen hin untersucht, die zu einer Überexpression bzw. Repression von Proteinen führen können, die mit einer CZA-Resistenz in Verbindung gebracht wurden: β-Laktamase-kodierende Gene wie z.B. *bla*$_{PDC}$ und dessen Regulatorgene (*bla*$_{AmpD}$, *bla*$_{AmpR}$, *bla*$_{AmpG}$); andere Gene wie *creD*, *ftsI*, *dacB*, *bla*$_{poxB}$, *mexA*, *mexB*, deren Regulatorgene (*mexR*, *nalC*, *nalD*, *DnaJ*, *DnaK*) und Gene, die ATP-abhängige Clp-Proteasen kodieren.

In den untersuchten Isolaten waren Mutationen in den Genen, die für die Proteine AmpC/PDC, PoxB/OXA-50-like, NalC und CreD kodieren besonders häufig. Mit Ausnahme von S41, PBP3/FTSI und NalD konnten in allen Proteinen vorhergesagte Aminosäuren-Substitutionen beschrieben werden. In den MexAB-OprM Regulatorproteinen zeigte sich besonders häufig die G71E Substitution in NalC (in 77% [47/61] der Isolate) und die V126E Substitution in MexR (47,5%; 29/61). Des Weiteren konnten Mutationen, die zu Substitutionen in PoxB (95,1%; 58/61), im PDC/AmpD-System (82%; 50/61) und in PBP3 (9,8%; 6/61) führen, nachgewiesen werden. In keinem der Isolate wurden Mutationen, die zu Substitutionen in AmpG, DnaJ, DnaK und ATP-abhängigen Clp Proteasen führen, gezeigt werden.

Bei mehreren Isolaten des gleichen STs konnten identische Mutationen, die folglich zu identischen Substitutionen führen, festgestellt werden. So zeigten sich beispielsweise in allen ST235 Isolaten aus Kolumbien, Argentinien und Mexiko Substitutionen in AmpC/PDC (G1D, A71V, T79A, V179L, G365A), AmpG (A583T), AmpR (G283E, M288R) und AmpD (G148A). Ebenso konnten weitere Mutationen, in allen Isolaten des ST244, ST309 und ST575 nachgewiesen werden.

Zusammenfassend kann festgehalten werden, dass 0,8% (18/2.235) aller untersuchten Enterobakterien und 22% (108/492) aller *P. aeruginosa* Stämme, die zwischen 2016 und 2017 aus klinischen Proben aus fünf lateinamerikanischen Ländern isoliert wurden, gegenüber CZA resistent waren. Unter den Enterobakterien wurde diese Resistenz durch MBL verursacht, während die möglichen Resistenzmechanismen bei *P. aeruginosa* vielfältig waren: MBL, weitere β-Laktamasen, Mutationen in β-Laktamasen, Mutationen in Effluxpumpen, im PDC/AmpD-System und in PBP3.

In Bezug auf die Empfindlichkeit der Enterobakterien wurden in anderen Studien ähnliche Resistenzraten beschrieben. In einer Studie, die 9.459 klinische Isolate aus sechs lateinamerikanischen Ländern, die zwischen 2012 und 2015 isoliert wurden, zeigte sich 0,2% aller Enterobakterien und 13,6% aller *P. aeruginosa* Stämme resistent gegenüber CZA (16). Obwohl die Zahlen

ähnlich sind, ist die von uns beschrieben CZA-Resistenz unter *P. aeruginosa* höher.

Die meisten der CZA-resistenten *P. aeruginosa* Isolate, bei welchen mittels PCR kein MBL-kodierendes Gen nachgewiesen werden konnten, gehörten zu den ST235, ST244 und ST111, welche als sogenannte „high-risk clones“ gelten. In Kolumbien wurde eine Assoziation zwischen dem ST235 und der Dissemination von *bla*$_{KPC-2}$ beschrieben (17). Interessanterweise wurden alle sieben Isolate bei denen *bla*$_{KPC-2}$ nachgewiesen werden konnte in kolumbianischen Gesundheitseinrichtungen isoliert und gehörten diesem ST an. Avibactam zeigt eine starke Hemmaktivität gegenüber KPC, jedoch wurden Substitutionen im Ω-Loop des Enzyms beschrieben, die zu einer reduzierten Empfindlichkeit gegenüber CZA führen. Bei den untersuchten Isolaten konnten keine Mutationen in *bla*$_{KPC-2}$ nachgewiesen werden, allerdings zeigten sich bei sechs der Isolate Mutationen in *nalD*, einem Gen, welches für einen Regulator der Effluxpumpe MexAB-OprM kodiert. Es wurde beschrieben, dass Mutationen in *nalD* zu einer verminderten Empfindlichkeit gegenüber CZA führen (18,19).

Obwohl Avibactam ebenfalls eine potente Hemmaktivität gegen PDC, ein Enzym der Ambler Klasse C aufweist, können Mutationen in *bla*$_{PDC}$ zu einer reduzierten Aktivität von CZA beitragen (20). In den analysierten Isolaten konnten 14 verschiedene Varianten von *bla*$_{PDC}$ nachgewiesen werden: Am häufigsten waren *bla*$_{PDC-3}$, *bla*$_{PDC-35}$ und *bla*$_{PDC-1}$. Keine dieser Varianten wurde bisher mit einer verminderten CZA-Empfindlichkeit in Verbindung gebracht. Weitere Analysen sind erforderlich, um die Beziehung zwischen den in dieser Studie identifizierten PDC-Varianten und der CZA-Resistenz in *P. aeruginosa* aufzuklären.

Gegenüber einigen ESBLs, wie z.B. Varianten der Familie PER und GES weist Avibactam eine geringere Hemmaktivität auf. Eine Verbindung zwischen diesen Enzymen und einer CZA-Resistenz in *P. aeruginosa* und Enterobakterien wurde beschrieben, wobei weitere Resistenzmechanismen, wie z.B. eine verringerte Permeabilität der Zellmembran möglicherweise eine Rolle spielen (21,22). Bei jeweils einem CZA-resistenten Isolat aus Argentinien und Chile konnte *bla*$_{PER-1}$ bzw. *bla*$_{PER-3}$ nachgewiesen werden. In Mexiko wurde eine hohe Prävalenz der

ESBL GES-19, sowie der Carbapenemase GES-20 in *P. aeruginosa* Stämmen beschrieben (23). In unserer Studie konnte in fünf mexikanischen Isolaten *bla*$_{GES-19}$ nachgewiesen werden, drei davon gehörten zum ST235. Zusätzlich konnte bei einem der Isolate des ST309 eine Tandemkonfiguration aus *bla*$_{GES-20}$ und *bla*$_{GES-19}$ nachgewiesen werden. Eine ähnliche Tandemkonfiguration aus *bla*$_{GES-19}$ und *bla*$_{GES-26}$ in zwei *P. aeruginosa* Isolaten desselben ST wurde bereits mit einer Resistenz gegenüber allen β-Laktamen assoziiert (24).

PBPs spielen eine fundamentale Rolle in der Integration der bakteriellen Zellwand. Der primäre Wirkungsmechanismus von β-Laktamen beruht auf der Hemmung dieser Proteine durch eine kovalente Bindung. Zahlreiche β-Laktame, die zur Therapie von gramnegativen Infektionen eingesetzt werden, haben eine hohe Affinität gegenüber FtsI (PBP3), welche auch das vorrangige pharmakologische Ziel von Ceftazidim ist (25,26). Die Substitutionen R504C und P527S sind mit einer verminderten Aktivität vieler β-Laktame assoziiert, dies betrifft auch Ceftazidim (25). In keinem der analysierten Isolate zeigten sich Mutationen, die zu diesen Substitutionen führen, allerdings beobachteten wir bei sechs Stämmen eine N117S Substitution in FtsI. Diese Variante wurde bisher nicht mit einer CZA-Resistenz in Verbindung gebracht und aufgrund der Position dieser Substitution innerhalb des Proteins ist eine Auswirkung auf die Aktivität von CZA unwahrscheinlich. Bemerkenswerterweise wurde diese Substitution ausschließlich bei Isolaten des ST309 aus Mexiko, Kolumbien und Chile festgestellt.

Effluxpumpen sind Proteinkomplexe, die Substanzen aus einer Zelle befördern. Durch den Hinaustransport von Antibiotika können diese zu einer geringen Empfindlichkeit bzw. zu Resistenzen gegenüber diesen Substanzen führen. Die Überexpression des Effluxsystems MexAB-OprM war in einer Studie mit einer CZA-Resistenz assoziiert (27). Mutationen, die zur Fehlfunktion von MexR, einem Negativregulator von MexAB-OprM, führen, bewirken eine erhöhte Expression dieses Effluxsystems, was wiederum zu höheren MIC von CZA führt. Wir konnten neun Isolate des ST575 mit Mutationen in MexR identifizieren; alle neun Stämme wurden in Mexiko isoliert. Wie bereits zuvor erwähnt, wurden bei mehreren Isolaten Mutationen in *nalD* nachgewiesen. NalD ist ein Negativregulator von MexAB. Mutationen in dem dafür kodierenden Gen

wurden mit einer Überexpression von MexAB und mit einer verminderten Empfindlichkeit gegenüber allen β-Laktamen assoziiert (19). Neben den sechs Isolaten, die *bla*$_{KPC-2}$ beherbergten, konnten Mutationen in *nalD* in zwölf weiteren Isolaten aus verschiedenen Regionen identifiziert werden.

Fazit

Unsere Beobachtungen in vitro legen nahe, dass CZA auch in Lateinamerika ein wichtiger Bestandteil des therapeutischen Arsenals im Kampf gegen Carbapenem-resistente gramnegative Erreger ist. Die Resistenz gegen CZA in Enterobakterien ist gering und hauptsächlich durch die Expression von MBL zu erklären. Bei *P. aeruginosa* ist die Rate der CZA-Resistenz höher und ist durch verschiedene enzymatische und nicht-enzymatische Mechanismen zu erklären. Einige der von uns beschriebenen Mutationen wurden bisher nicht mit CZA-Resistenz in Verbindung gebracht, weswegen weitere Studien erforderlich sind.

Unsere Ergebnisse unterstreichen die Bedeutung der molekularen Diagnostik in der klinischen Mikrobiologie, sowie die wichtige Rolle von Antibiotika zur Therapie von MBL-exprimierenden und anderen Carbapenem-resistenten gramnegativen Mikroorganismen. Darüber hinaus zeigt die Resistenz gegenüber Reserveantibiotika wie Carbapenemen, Polymyxinen und CZA die Notwendigkeit eines rationalen Gebrauchs von Antiinfektiva.

Im Zusammenhang mit multiresistenten gramnegativen Erregern untersuchten wir in weiteren Arbeiten unter anderem Mechanismen, die zur Resistenz gegenüber Colistin in Carbapenem-resistenten *K. pneumoniae* Isolaten führen und charakterisierten CTX-M-Group-1 produzierende *E. coli* Isolate (28,29). Des Weiteren untersuchten und verglichen wir diagnostische Methoden zum Nachweis von molekularen Resistenzdeterminanten wie β-Laktamase-kodierende Gene und *vanA/B*, sowie mikrobiologische Methoden zur Testung einer Fosfomycin-Empfindlichkeit (30,31). Zuletzt untersuchten wir den Einfluss von Antibiotic Stewardship Programmen auf den Verbrauch von Antibiotika und Antibiotikaresistenzen in vier kolumbianischen Gesundheitseinrichtungen (32).

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In vitro susceptibility to ceftazidime/avibactam and comparators in clinical isolates of *Enterobacterales* from five Latin American countries

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Abstract

Background: High rates of resistance to third generation cephalosporins and carbapenems in *Enterobacterales* have been reported in Latin America. Ceftazidime/avibactam (CZA) is the combination of a third-generation cephalosporin and a non-β-lactam β-lactamase inhibitor, which has shown activity against isolates producing class A, C and D β-lactamases. Herein, we evaluated the activity of CZA and comparators against clinical isolates of *Enterobacterales* in Latin America.

Methods: The activity of CZA and comparators was evaluated against clinical isolates of *Enterobacterales* from Argentina, Brazil, Chile, Colombia and Mexico that were collected between January 2016 and October 2017. One specific phenotypic subset was evaluated. A carbapenem non-susceptible (CNS) phenotype was defined as any isolate displaying a minimum inhibitory concentration (MIC) ≥1 mg/L for ertapenem.

Results: CZA was active against 95.8% of all isolates and 77.5% of CNS isolates. Fosfomycin (FOS) and tigecycline (TGC) were the second most active antibiotics with 93.4% of *Enterobacterales* being susceptible.

Conclusions: The results of this study underline the potential therapeutic role of CZA in Latin America.

Keywords: Antimicrobial activity; Argentina; Brazil; Chile; Colombia; Mexico

Introduction

Antimicrobial resistance is a threat to public health. *Enterobacterales* are some of the most common and pathogenic microorganisms that have acquired resistance to several classes of antimicrobials [1]. Particularly concerning is the resistance to carbapenems since these agents are often considered the last resort antibiotics. In addition, infections caused by carbapenem-resistant enterobacteria are associated with higher costs and mortality rates [2,3].

The most frequently found carbapenem resistance mechanism is the production of carbapenemases, among which *Klebsiella pneumoniae* carbapenemases

(KPC) are the most widely distributed worldwide and are endemic in several countries of the Latin American region [4]. Ceftazidime/avibactam (CZA) is the combination of a third-generation cephalosporin and a non-β-lactam inhibitor capable of inhibiting several class D, C and A β-lactamases, including the KPC-family enzymes. Several in vitro, in vivo and clinical studies have reported favorable results with CZA against carbapenemase-producing enterobacteria, while being less toxic than other agents commonly used to treat carbapenem-resistant bacteria, such as colistin and aminoglycosides [5,6,7].

Herein, we evaluated the activity of CZA and comparators against 2252 clinical isolates of *Enterobacterales* from 20 healthcare institutions located in Argentina, Brazil, Chile, Colombia, and Mexico between January 2016 and October 2017.

Results

The distribution of the 2252 isolates of *Enterobacterales* per country and species is shown in Table 1. Overall, 95.8% (2158/2252) of the isolates were susceptible to CZA (minimum inhibitory concentration of 90% of isolates (MIC90) ≤1 mg/L). The highest susceptibility was observed in *Escherichia coli* (97.9%), followed by *Serratia marcescens* (94.5%), *Klebsiella aerogenes* (93.3%), *Klebsiella pneumoniae* (92.1%) and isolates of the *Enterobacter cloacae* complex with a susceptibility of 92.0% (Table 2). Fosfomycin (FOS) and tigecycline (TGC) were the second most active antibiotics with 93.4% of *Enterobacterales* susceptible, followed by the carbapenems meropenem (MEM) (88.7%), imipenem (IMI) (87.1%) and ertapenem (ETP) (82.4%).

Table 1. Susceptibility of *Enterobacterales* to ceftazidime/avibactam and comparators by country.

Microorganism	Number of isolates	Percentage of susceptibility								
		CZA	CAZ	FEP	TZP	ETP	IMI	MEM	TGC	FOS
Argentina	233									
E. coli	160	98	54	91	60	96	96	97	98	98
CNS	7	57	0	14	14	-	14	29	29	57
K. pneumoniae	65	99	52	62	49	82	88	89	94	97
CNS	12	100	8,3	8,3	8,3	-	33	42	75	92
E. cloacae complex	4	100	75	75	75	75	75	75	75	75
CNS	0									
S. marcesens	4	75	75	75	75	75	75	75	75	75
CNS	1	0	0	0	0	-	0	0	0	0
Brazil	85									
E. coli	20	95	65	65	80	70	75	75	90	100
CNS	6	83	14	14	43	-	14	14	57	86
K. pneumoniae	23	87	4,3	8,7	13	22	17	22	74	96
CNS	18	83	0	0	0	-	0	0	67	94
E. cloacae complex	24	100	25	29	58	63	83	88	79	79
CNS	9	100	0	11	44	-	67	56	67	78
S. marcesens	18	100	100	61	67	83	89	83	89	83
CNS	2	100	0	0	0	-	0	0	50	100
Chile	443									
E. coli	347	99	70	77	91	89	94	97	95	95
CNS	39	95	23	26	51	-	54	69	59	92
K. pneumoniae	66	99	44	52	61	79	91	83	94	91
CNS	14	93	0	0	14	-	57	21	93	71
E. cloacae complex	21	100	81	100	91	91	100	100	95	86
CNS	2	100	100	100	50	-	100	100	100	100
S. marcesens	9	100	67	67	78	67	100	89	100	100
CNS	3	100	33	33	33	-	100	67	100	100
Colombia	1396									
E. coli	813	97	79	82	91	91	95	95	96	94
CNS	76	72	0	0	0	-	45	47	65	76
K. pneumoniae	441	90	52	57	62	69	74	76	91	92
CNS	137	69	0	0	0	-	18	23	73	80
E. cloacae complex	82	88	48	48	55	59	78	78	90	81
CNS	32	74	12	12	21	-	29	38	77	71
S. marcesens	60	93	62	63	63	65	67	73	77	93
CNS	21	81	4,8	4,8	19	-	19	24	52	81
Mexico	95									
E. coli	69	100	35	39	74	87	91	97	96	94
CNS	9	100	11	0	11	-	44	78	67	11
K. pneumoniae	15	100	67	67	40	87	87	87	100	100
CNS	2	100	0	0	0	-	50	50	100	100
E. cloacae complex	11	91	27	18	9,1	64	18	73	91	100
CNS	4	75	0	0	0	-	25	25	100	100

Table 2. Susceptibility of Enterobacterales to ceftazidime/avibactam according to minimum inhibitory concentration (MIC) (mg/L) distribution and susceptibility to comparators.

		Ceftazidime/avibactam											Susceptibility to comparators (% isolates susceptible)							
		Cumulative percantage of isolates at each MIC (mg/L)																		
Microorganism	Number of isolates	≤1	2	4	8	16	32	64	≥128	MIC_{50}	MIC_{90}	%S	CAZ	FEP	TZP	ETP	IMI	MEM	TGC	FOS
Enterobacterales	2252	89	94	95	96	96	97	98	100	≤1	2	96	64	68	79	82	87	89	93	93
CNS	396	45	66,9	75	78	79	83	88	100	2	≥128	78	8,6	12	27	-	32	36	69	81
E. coli	1409	93	97	98	98	98	99	99	100	≤1	≤1	98	72	76	90	90	92	96	96	95
CNS	137	44	70	77	80	82	85	89	100	2	≥128	80	17	22	47	-	45	53	61	83
K. pneumoniae	610	82	88	91	92	92	94	95	100	≤1	4	92	50	53	59	70	76	77	91	93
CNS	183	45	63	72	74	75	80	85	100	2	≥128	74	2,7	4,9	14	-	21	22	74	82
E. cloacae complex	112	80	88	90	92	92	97	97	100	≤1	4	92	43	46	52	63	71	80	80	80
CNS	41	46	71	76	81	81	90	93	100	2	32	81	7,3	15	20	-	39	44	81	73
K. aerogenes	30	87	90	93	93	97	97	100	100	≤1	2	93	67	70	83	73	83	83	90	97
CNS	8	50	63	75	75	88	88	100	100	≤1	64	75	13	13	50	-	38	38	50	75
S. marcescens	91	81	92	95	95	95	95	96	100	≤1	2	95	63	65	69	70	74	78	80	95
CNS	27	41	74	81	81	81	81	85	100	2	≥128	82	7,4	7,4	19	-	26	26	56	82

In all five countries, the susceptibility of *Enterobacterales* to CZA was similarly high, ranging from 99.1% in Chile (MIC_{90} ≤ 1 mg/L), 98.9% in Mexico (MIC_{90} ≤ 1 mg/L), 97.4% in Argentina (MIC_{90} ≤ 1 mg/L), 96.5% in Brazil (minimum inhibitory concentration of 50% of isolates (MIC_{50}) ≤ 1 mg/L, MIC_{90} 2 mg/L) to 94.3% in Colombia (MIC_{50} ≤ 1 mg/L, MIC_{90} 2 mg/L). Comparable results were observed for FOS (92.5%–97.4%) and TGC (81.5%–95.8%). For carbapenem non-susceptible (CNS) *Enterobacterales*, CZA was active against 77.5% of all tested strains (MIC_{50} 2 mg/L, MIC_{90} ≥ 128 mg/L). The activity of CZA was the highest in CNS isolates from Chile (94.8%, MIC_{50} 2 mg/L, MIC_{90} 8 mg/L), followed by Mexico (93.3%, MIC_{50} ≤ 1 mg/L, MIC_{90} 1 mg/L), Brazil (88.6%, MIC_{50} ≤ 1 mg/L, MIC_{90} 32 mg/L), Argentina (80%, MIC_{50} ≤ 1 mg/L, MIC_{90} 64 mg/L), and Colombia (71.3%, MIC_{50} 2 mg/L, MIC_{90} ≥ 128 mg/L) (**Table 1**).

For all species of *Enterobacterales*, regardless of their susceptibility profile, CZA was the compound with the highest activity when compared with other β-lactam agents. For isolates of *E. coli* and *E. cloacae* complex, CZA was superior to all other antimicrobials tested. In the case of *K. pneumoniae* and *K. aerogenes*, the activity of FOS was slightly superior to CZA, whereas for *S. marcescens* both antimicrobials showed a susceptibility of 94.5%.

From the 2252 isolates tested, 396 (17.6%) were found to be CNS; of note, 46.2% were identified as *K. pneumoniae*. CZA was active against 77.5% of the CNS isolates (MIC_{50} 2 mg/L, MIC_{90} ≥ 128 mg/L), with the highest activity against *S. marcescens* (81.5%), while the lowest susceptibility was observed for *K. pneumoniae* (74.3%). For this group, the activity of CZA was superior to all β-lactams and superior or equal to that of FOS for isolates of *E. cloacae* complex, *K. aerogenes* and *S. marcescens*.

Discussion

This study showed that 95.8% of clinical isolates of *Enterobacterales* from five Latin American countries, collected between January 2016 and October 2017, were susceptible to CZA (MIC_{90} ≤ 1 mg/L). The susceptibility to CZA between species ranged from 97.9% for *E. coli* to 92.0% for isolates of *E. cloacae* complex. Furthermore, 77.5% of CNS isolates remained susceptible to CZA.

These results underline the potential therapeutic role of CZA for patients infected with KPC-producing and other carbapenemase-producing enterobacteria, which are prevalent in the Latin American region [4,7].

Although the present study might be limited by the small number of isolates from Mexico and Brazil and the fact that they are from a single center in Argentina, Brazil and Mexico, our results are similar to most reports described previously by other authors. In a study by Flamm et al. [8], CZA was evaluated against 130 clinical urinary isolates of *Enterobacterales* collected in 2011 from Argentina, Brazil, Chile, Colombia, Mexico, Panama, and Venezuela, finding a MIC_{90} of 0.25 mg/L. Of the evaluated strains, 0.8% were resistant to MEM. Similarly, Karlowsky et al. [9] evaluated the activity of CZA and comparators against clinical isolates of *Enterobacterales* and *P. aeruginosa* collected between 2012 and 2015 from six Latin American countries (Argentina, Brazil, Chile, Colombia, Mexico and Venezuela). In this study, CZA was active against 99.7% of 7665 *Enterobacterales*, which is similar to our findings. Furthermore, 5.1% of all isolates were carbapenem (MEM) non-susceptible. In the MEM non-susceptible subgroup, the authors observed that CZA was active against 95.4% of isolates, which is significantly higher compared to our observations.

The differences in CZA susceptibility of the non-susceptible subgroups could be explained by the different hospitals and geographical areas included in the study, as well as the changes in the epidemiology of resistance mechanisms between the study periods. For example, in the case of Brazil, susceptibility rates to CZA in this study were inferior to those observed previously against *K. pneumoniae* isolates in a surveillance study by Rossi et al. (100% susceptible) [10]. An increase in class B β-lactamases (which were detected in 0.2% of all *Enterobacterales* by Karlowsky et al.) or the emergence of different mechanisms of resistance to CZA in class A β-lactamase-producing *K. pneumoniae* as reported in the literature could explain this difference [11,12].

Materials and methods

Isolates were collected in each of the participating institutions between January 2016 and October 2017. Upon reception, species confirmation was performed

using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Biomeriéux, Marcy-l'Étoile, France). Susceptibility testing was performed in the laboratory of the research group Resistencia Antimicrobiana y Epidemiología Hospitalaria (RAEH), Universidad El Bosque, Bogotá, Colombia. Minimum inhibitory concentrations (MICs) were determined by broth microdilution using customized Sensititre plates (TREK Diagnostic Systems, East Grinstead, West Sussex, UK), with *E. coli* ATCC 25922 as quality control, following Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Antibiotics evaluated included: ceftazidime/avibactam (CZA; 1/4–128/4 mg/L), ceftazidime (CAZ; 2–32 mg/L), cefepime (FEP; 2–64 mg/L), piperacillin/tazobactam (TZP; 2/4–128/4 mg/L), ertapenem (ETP; 0.25–32 mg/L), imipenem (IMP; 0.25–128 mg/L), meropenem (MEM; 0.25–128 mg/L), tigecycline (TGC; 0.25–8 mg/L) and fosfomycin (FOS; 8–128 mg/L). With the exception of FOS and TGC, results were interpreted according to the CLSI 2018 breakpoints [14]. FOS breakpoints for *Enterobacterales* were extrapolated from the *E. coli* breakpoint by CLSI (FOS non-susceptible MIC ≥128 mg/L). United States Food and Drug Administration product package insert criteria were used as breakpoints for TGC (susceptible: ≤2 mg/L; intermediate: 4 mg/L; resistant: ≥8 mg/L) [15]. The specific phenotypic subset defined as a carbapenem non-susceptible (CNS) phenotype included isolates displaying a MIC ≥1 mg/L for ETP.

Conclusions

We report excellent activity of CZA against diverse *Enterobacterales* collected in Latin America. The lower rates of CZA susceptibility among CNS isolates in our study highlights the importance of active surveillance programs in order to follow the evolution of resistance mechanisms against the antibiotic armamentarium, including newly introduced antimicrobial agents.

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Evaluation of in vitro susceptibility to ceftazidime/avibactam of clinical isolates of carbapenem non-susceptible gram-negative bacilli from Colombia

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Abstract

Background: Ceftazidime/avibactam (CZA) is a novel combination of a third-generation cephalosporin and a diazabicyclooctane β-lactamase inhibitor, which has been shown to be active against a broad range of class A, C and D β-lactamases. In Colombia, high rates of multidrug-resistant *Enterobacterales* (Ent) and *P. aeruginosa* (Pae) have been reported. Of special concern are KPC enzymes endemic in Ent and found in Pae, which are associated with higher mortality and healthcare costs, as well as limited therapeutic options. Herein, we evaluate the susceptibility of clinical isolates of carbapenem non-susceptible Ent (CNS-E) and Pae (CNS-P) to CZA with the aim of understanding its role as a therapeutic option for these bacteria.

Methods: Three hundred ninety-nine non-duplicate clinical isolates of carbapenem non-susceptible gram-negative bacilli were collected in 13 medical centers from twelve Colombian cities, from January 2016 to October 2017 (137 *K. pneumoniae* [Kpn], 76 *E. coli*, 34 *Enterobacter* spp., 21 *S. marcescens* [Sma] and 131 Pae). CNS-E was defined as minimum inhibitory concentrations (MIC) ≥ 1 mg/L for ertapenem and CNS-P was defined as MIC ≥ 4 mg/L for meropenem. MIC were determined by broth microdilution and interpreted according to current CLSI guidelines. CZA MIC were determined using double dilutions of ceftazidime and a fixed concentration of avibactam of 4 mg/L. Comparator agents were ceftazidime, cefepime, piperacillin/tazobactam, imipenem, meropenem, tigecycline (TGC) and fosfomycin (FOS).

Results: Antimicrobial activity of CZA and comparators is shown in table 1. CZA susceptibility ranged from 68.9% in Kpn to 81.0% in Sma, whereas 45% of Pae susceptible to CZA. In both, CNS-E and CNS-P, CZA was superior to all other tested β-lactam compounds. Notably, in CNS-E CZA susceptibility was comparable to FOS and TGC (except for TGC in Sma).

Conclusions: Our findings indicate that CZA is superior to all other β-lactams in clinical isolates of CNS-E. CNS-P isolates were CZA susceptible in a lesser degree, suggesting the presence of resistance mechanisms different to class A enzymes. Nevertheless, CZA was superior to all other tested β-lactams. Our results highlight the key role that new antibiotics such as CZA play in KPC endemic countries like Colombia.

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Evaluation of *in vitro* susceptibility to ceftazidime/avibactam of clinical isolates of carbapenem non-susceptible gram-negative bacilli from Colombia

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Abstract

Background: Ceftazidime/avibactam (CZA) is a novel combination of a third-generation cephalosporin and a diazabicyclooctane β-lactamase inhibitor, which has been shown to be active against a broad range of class A, C and D β-lactamases. In Colombia, high rates of multidrug-resistant Enterobacterales (Ent) and *P. aeruginosa* (*Pae*) have been reported. Of special concern are KPC enzymes endemic in Ent and found in *Pae*, which are associated with higher mortality and healthcare costs, as well as limited therapeutic options. Herein, we evaluate the susceptibility of clinical isolates of carbapenem non-susceptible Ent (CNS-E) and *Pae* (CNS-P) to CZA with the aim of understanding its role as a therapeutic option for these bacteria.

Methods: Three hundred ninety-nine non-duplicate clinical isolates of carbapenem non-susceptible gram-negative bacilli were collected in 13 medical centers from twelve Colombian cities, from January 2016 to October 2017 (137 *K. pneumoniae* [*Kpn*], 76 *E. coli*, 34 *Enterobacter spp.*, 21 *S. marcescens* [*Sma*] and 131 *Pae*). CNS-E was defined as minimum inhibitory concentrations (MIC) ≥ 1 mg/L for ertapenem and CNS-P was defined as MIC ≥ 4 mg/L for meropenem. MIC were determined by broth microdilution and interpreted according to current CLSI guidelines. CZA MIC were determined using double dilutions of ceftazidime and a fixed concentration of avibactam of 4 mg/L. Comparator agents were ceftazidime, cefepime, piperacillin/tazobactam, imipenem, meropenem, tigecycline (TGC) and fosfomycin (FOS).

Results: Antimicrobial activity of CZA and comparators is shown in table 1. CZA susceptibility ranged from 68.9% in *Kpn* to 81.0% in *Sma*, whereas 45% of *Pae* susceptible to CZA. In both, CNS-E and CNS-P, CZA was superior to all other tested β-lactam compounds. Notably, in CNS-E CZA susceptibility was comparable to FOS and TGC (except for TGC in *Sma*).

Conclusions: Our findings indicate that CZA is superior to all other β-lactams in clinical isolates of CNS-E. CNS-P isolates were CZA susceptible in a lesser degree, suggesting the presence of resistance mechanisms different to class A enzymes. Nevertheless, CZA was superior to all other tested β-lactams. Our results highlight the key role that new antibiotics such as CZA play in KPC endemic countries like Colombia.

Background

- In Colombia, high rates of multidrug-resistant Enterobacterales (Ent) and *P. aeruginosa* (*Pae*) have been reported (1).
- KPC enzymes in Colombia are endemic in Ent and found in *Pae* (1)
- Carbapenemases are associated with higher mortality rates and healthcare costs (2,3). There are few therapeutic options, which are frequently toxic (4).
- Ceftazidime/avibactam (CZA) is a "game changer" combination (4; **Figure 1**).

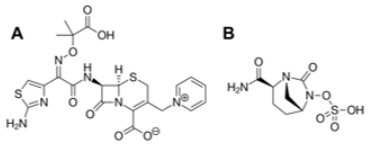
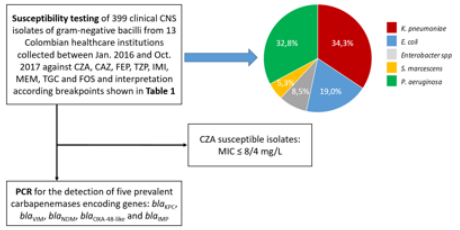


Figure 1. Chemical structures of **A.** Ceftazidime, a 3rd Gen cephalosporin and **B.** Avibactam, a diazabicyclooctane (DBO) inhibitor of class A, C and D β-lactamases

AIM: To evaluate the susceptibility to CZA in carbapenem non-susceptible Ent and *Pae* from Colombia

Methods



CZA: ceftazidime/avibactam, CAZ: ceftazidime, FEP: cefepime, TZP: piperacillin/tazobactam, IMI: imipenem, MEM: meropenem, TGC: tigecycline, FOS: fosfomycin

Carbapenem non-susceptibility (CNS) was defined as MIC ≥ 1 mg/L for ertapenem in Ent and MIC ≥ 4 mg/L for MEM in *Pae*.

Table 1. Non-susceptibility definitions used in this study (in mg/L)

All breakpoints were interpreted according to CLSI 2018, unless indicated otherwise

Microorganism	CZA	CAZ	FEP	TZP	IMI	MEM	TGC	FOS
Enterobacterales	≥ 16/4	≥ 8	≥ 4	≥ 32/4	≥ 2	≥ 2	≥ 4[a]	≥ 128[b]
P. aeruginosa	≥ 16/4	≥ 16	≥ 16	≥ 32/4	≥ 4	-	-	(≥ 128)[c]

[a] USA-FDA product package insert criteria were used as breakpoints for tigecycline.
[b] FOS breakpoints for Ent were extrapolated from *E. coli* breakpoints by CLSI (fosfomycin non-susceptible MIC ≥ 128 mg/L).
[c] There are no fosfomycin breakpoint for *Pae* by EUCAST or CLSI. ECOFF of ≤ 128 µg/ml by EUCAST for *P. aeruginosa* was applied.

Results

Susceptibility testing revealed (Table 2):

- In CNS Ent, CZA was superior to all tested β-lactam compounds.
- Distribution of CNS Ent isolates according to CZA MIC distribution is shown in **Figure 2**.
- In CNS Ent, CZA susceptibility was comparable to FOS and TGC (except TGC in *Sma*).
- Similarly, CZA was superior to all tested β-lactam compounds in CNS *Pae*, with 45% susceptibility, followed by TZP, FEP and CAZ.
- Distribution of CNS *Pae* isolates according to CZA MIC distribution is shown in **Figure 3.**

Results

Table 2. Percentage of *in vitro* susceptibility of 399 clinical isolates of carbapenem non-susceptible gram-negative bacilli to CZA and comparators

Species	Number of isolates	Susceptible Isolates (%)							
		CZA	CAZ	FEP	TZP	IMI	MEM	TGC	FOS
K. pneumoniae	137	69	0	0	0	18	23	73	80
E. coli	76	72	0	0	0	45	47	64	76
Enterobacter spp.	34	74	12	12	21	29	38	74	71
S. marcescens	21	81	5	5	19	19	24	52	81
P. aeruginosa	131	45	27	29	30	1	-	-	81

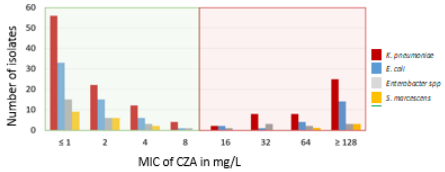


Figure 2. Distribution of carbapenem non-susceptible Ent isolates according to CZA MIC distribution

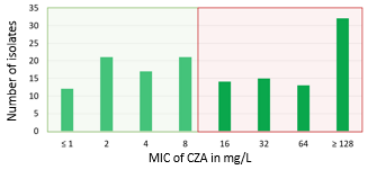


Figure 3. Distribution of carbapenem non-susceptible *Pae* isolates according to CZA MIC distribution

PCR of 151 CZA resistant strains revealed (Table 3):

- In 50.9% and 30.6% of all CZA resistant Ent and *Pae*, respectively, no carbapenemase encoding gene was detected
- bla_{KPC} alone was detected in 30.4% of *Ent* and 36.1% of *Pae*
- The presence of bla_{KPC} and genes encoding for class B carbapenemases, was detected in 9.3% of *Kpn*, 10% of *Enterobacter* spp. and 8.3% of *Pae*
- Genes encoding for class B carbapenemases were detected in 38.9% of *Pae* and 19.0% of Ent isolates
- None of the analyzed isolates carried bla_{IMP} or $bla_{OXA-48-like}$

Results

Table 3. Results of PCR targeting five carbapenemase encoding genes of 151 CZA resistant gram-negative bacilli

Species	Number of isolates	Positive Isolates PCR (%)				
		bla_{KPC}	bla_{VIM}	bla_{NDM}	bla_{KPC}+bla_{VIM}	bla_{KPC}+bla_{NDM}
K. pneumoniae	43	0 (0)	0 (0)	10 (23,3)	0 (0)	3 (7)
E. coli	22	0 (0)	0 (0)	1 (4,5)	0 (0)	0 (0)
Enterobacter spp.	10	0 (0)	1 (10)	1 (10)	0 (0)	1 (10)
S. marcescens	4	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)
P. aeruginosa	72	20 (27,8)	22 (30,6)	0 (0)	6 (8,3)	0 (0)
Total	151	20 (13,2)	23 (15,2)	13 (8,6)	6 (4)	4 (2,6)

Conclusions

- CZA had the highest antimicrobial activity compared to β-lactam compounds against clinical isolates of Ent and *Pae* from Colombia.
- The high number of CZA resistant isolates in which no carbapenemase encoding gene was detected highlight the importance of non-enzymatic mechanisms and/or other β-lactamases not included in this study.
- Isolates in which only bla_{KPC} was detected require further studies to rule out additional resistance mechanisms and/or mutations in the KPC-encoding gene.
- Further studies are required to establish resistance mechanisms in strains in which no class B carbapenemase encoding gene was detected.

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Molecular mechanisms of resistance to ceftazidime/avibactam in clinical isolates of *Enterobacterales* and *Pseudomonas aeruginosa* in Latin American hospitals

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Abstract

Ceftazidime-avibactam (CZA) is the combination of a third-generation cephalosporin and a new non-β-lactam β-lactamase inhibitor capable of inactivating class A, C, and some D β-lactamases. From a collection of 2,727 clinical isolates of *Enterobacterales* (*n* = 2,235) and *P. aeruginosa* (*n* = 492) that were collected between 2016 and 2017 from five Latin American countries, we investigated the molecular resistance mechanisms to CZA of 127 (18/2,235 [0.8%] *Enterobacterales* and 109/492 [22.1%] *P. aeruginosa*). First, by qPCR for the presence of genes encoding KPC, NDM, VIM, IMP, OXA-48-like, and SPM-1 carbapenemases, and second, by whole-genome sequencing (WGS). From the CZA-resistant isolates, MBL-encoding genes were detected in all 18 *Enterobacterales* and 42/109 *P. aeruginosa* isolates, explaining their resistant phenotype. Resistant isolates that yielded a negative qPCR result for any of the MBL encoding genes were subjected to WGS. The WGS analysis of the 67 remaining *P. aeruginosa* isolates showed mutations in genes previously associated with reduced susceptibility to CZA, such as those involved in the MexAB-OprM efflux pump and AmpC (PDC) hyperproduction, PoxB (*bla*$_{\text{OXA-50-like}}$), FtsI (PBP3), DacB (PBP4), and OprD. The results presented here offer a snapshot of the molecular epidemiological landscape for CZA resistance before the introduction of this antibiotic into the Latin American market. Therefore, these results serve as a valuable comparison tool to trace the evolution of the resistance to CZA in this carbapenemase-endemic geographical region.

Introduction

Enterobacterales and the nonfermenting bacilli *P. aeruginosa* are among the most common pathogenic microorganisms that have acquired resistance to several antibiotic classes (1). The dissemination of β-lactam resistance determinants among these bacteria has radically decreased the effectiveness of last-generation β-lactams, including cephalosporins, carbapenems, and therapeutic combinations with β-lactamase inhibitors. The accumulation of resistance mechanisms to β-lactams and some other antibiotic families

significantly hinders the treatment of infections, and obliges the use of less effective and more toxic antibiotics such as colistin and aminoglycosides (1, 2).

The most effective resistance mechanism to carbapenems in Gram-negative pathogens is the production of carbapenemases. In *Enterobacterales*, many class A β-lactamase-encoding genes can yield a carbapenem resistant phenotype. However, *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ are the most common transmissible genes circulating worldwide and, notably, are endemic to some geographical areas such Latin America (3, 4). In *P. aeruginosa*, resistance to carbapenems can be achieved either by the hyperproduction of the chromosomal cephalosporinase AmpC or by the production of acquired carbapenemases, particularly of class B metallo-β-lactamases (MBL) such as VIM-2. In addition, nonenzymatic mechanisms such as the modification or inactivation of the porin OprD, or the upregulation of different chromosomally encoded efflux pumps, are also common (5–7).

In the last few years, novel β-lactams/β-lactamase inhibitor combinations are available for the treatment of infections caused by carbapenem resistant *Enterobacterales* and carbapenem resistant *P. aeruginosa* (8). Among them, ceftazidime-avibactam (CZA) is the combination of an extended-spectrum cephalosporin and a diazabicyclooctane (DBO)-based, non-β-lactam β-lactamase inhibitor. Avibactam is capable of inhibiting the majority of KPC enzymes, including the most wide-spread types, KPC-2 and KPC-3, in addition to other class A β-lactamases; class C cephalosporinases; and to a various degree class D β-lactamases, like some members of the OXA-48 family. However, avibactam cannot inhibit any class B MBL (9).

Resistance to CZA has been extensively reported (1, 2, 6, 10–13). Most cases of CZA resistance in *Enterobacterales*, especially in *Klebsiella pneumoniae*, have been associated with amino acid substitutions in KPC-2 and KPC-3, particularly the D179Y substitution in the Ω-loop (14–16). Recently, *K. pneumoniae* isolates resistant to CZA due to the production of KPC-31 (D179Y) and KPC-115 (L168P, ΔAsp169, ΔSer170) were reported causing an outbreak during the COVID-19 pandemic in Argentina (17). CZA resistance due to mutations in *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ has also been described

in *Enterobacterales* (18–20). For *P. aeruginosa* resistance to CZA is commonly associated with the presence of amino acid substitution in the Ω-loop of the pseudomonal-derived cephalosporinase (PDC), overexpression of PDC and genetic loss of *oprD*, overexpression of *bla*$_{\text{OXA-50-like}}$ β-lactamases, and duplication in the *bla*$_{\text{OXA-2}}$, which codes for OXA-539, among others (8). Furthermore, production of ESBLs such as PER-1, which particularly shows weaker kinetic inhibition constants for avibactam, and the presence of tandem *bla*$_{\text{GES-19}}$ and *bla*$_{\text{GES-26}}$, have been also associated with resistance to CZA (8, 21, 22).

Previously, our group determined the rates of susceptibility to CZA and other relevant antibiotics of clinical *Enterobacterales* isolates collected prior to the introduction of this antibiotic into the clinical practice in Latin America. The resistance rate found in that study was 4.2% (23). Herein, we reassess the phenotypic resistance to CZA of the 94 CZA-resistant *Enterobacterales* strains identified in that previous study; describe the phenotypic resistance rates to CZA of 492 *P. aeruginosa* clinical isolates collected between 2016 and 2017; and explore the molecular mechanisms leading to CZA resistance in these clinical isolates using whole-genome sequencing (WGS).

Results

Molecular characterization of CZA-resistant Enterobacterales

To compare the data previously published for the *Enterobacterales* collection with the new data on the *P. aeruginosa* isolates from this study, we checked the susceptibility to CZA of 94 isolates previously identified as CZA-resistant. However, after analyzing together the MIC data with Etest, only 18 isolates were confirmed to be truly CZA-resistant. Therefore, the updated CZA resistance rate of this collection of *Enterobacterales* is 0.8% (18/2,235). Of interest, all 18 CZA-resistant isolates were collected in Colombia, at different times, from nine medical centers located in nine cities. For *Enterobacterales*, we expanded the battery of tests performed before, adding the RAPIDEC Carba-NP assay to detect carbapenemase activity, and qPCR to confirm the presence of at least one MBL-encoding gene in these isolates (Table 1). Furthermore,

three isolates of *K. pneumoniae* and one *Enterobacter cloacae* complex co-harboring *bla*$_{NDM}$ and *bla*$_{KPC}$ were detected. Since resistance to CZA is explained by the presence of at least one MBL-encoding gene in CZA-resistant *Enterobacterales*, WGS was not performed on any of these isolates.

Antibiotic susceptibility and molecular characterization of CZA-resistant P. aeruginosa

The distribution of the 492 isolates of *P. aeruginosa* per country is shown in Table 2. Overall, 22.1% (109/492, MIC_{50} 4/4 mg/L, MIC_{90} 64/4 mg/L) of the isolates were resistant to CZA. In addition, complete MIC data are presented in Table S1.

All CZA-resistant *P. aeruginosa* isolates were then subjected to RAPIDEC Carba-NP test and qPCR. These tests found 42 isolates (38.5%) with MBL (three for *bla*$_{IMP}$; 31 for *bla*$_{VIM}$; one for *bla*$_{SPM-1}$; and seven carrying a combination of *bla*$_{KPC}$ and *bla*$_{VIM}$), and eight positives for *bla*$_{KPC}$. However, 59 did not carry any carbapenemases (Table 3). Notably, the only isolate harboring *bla*$_{SPM-1}$ yielded a negative result in the RAPIDEC Carba-NP assay.

WGS analysis of P. aeruginosa isolates resistant to CZA and associated resistance genes

A total of 67 *P. aeruginosa* genomes were sequenced. This number corresponds to the 59 isolates that yielded negative results for the multiplex qPCR and eight additional isolates that tested positive for the presence of *bla*$_{KPC}$. Due to unexpected low sequence coverage (<30×), we excluded six samples from the subsequent analysis (five isolates negative for any carbapenemase gene and one positive for *bla*$_{KPC}$). The remaining 61 samples showed quality values over 90%. We obtained between 110 to 137 contigs per isolate sequenced, with a length of the assemblies between 6.3 to 7.2 Kb and, a GC content ranging from 65.8% to 66.5%. Sequencing quality data are presented in Table S2.

WGS analysis revealed 23 known sequence types (STs), and five new STs, as shown in Fig. 1. Relevant STs found included ST111 (*n* = 1) and ST308 (*n* = 1) from Colombia; ST357 (*n* = 1) from Chile; and ST309 (*n* = 6) were found in four isolates from Mexico, one from Colombia, and one from Chile. Clonal dissemination was observed among some isolates: ST575 (*n* = 9) was only reported in Mexico; ST235 (*n* = 16) in Colombia, Mexico, Brazil, and Argentina; and ST244 mainly in Argentina.

Confirming their species identity, sequence analysis of the *P. aeruginosa* genomes showed that all of them carried *bla*$_{\text{AmpC}}$ and *bla*$_{\text{OXA-50-like}}$. From the 61 genomes analyzed, 17 (27.9%) harbored *bla*$_{\text{OXA-2}}$: 11 isolates belonging to the ST235 from Mexico and Colombia, four isolates with ST309 from Mexico, and two belonging to the ST308 and ST261 isolated from Colombia (Fig. 1). However, none of the evaluated isolates harbored mutations in *bla*$_{\text{OXA-2}}$, including duplication in the *bla*$_{\text{OXA-2}}$, which encodes for OXA-539.

Three isolates from Mexico belonging to the ST235 and one belonging to the ST30 harbored *bla*$_{\text{GES-19}}$. Interestingly, one isolate belonging to the ST309 from Mexico harbored *bla*$_{\text{GES-19}}$ and *bla*$_{\text{GES-20}}$ in tandem. Also, one isolate from Argentina and one from Chile, were found to harbor *bla*$_{\text{PER-1}}$ and *bla*$_{\text{PER-3}}$, respectively. All sequenced isolates harboring *bla*$_{\text{KPC-2}}$ (*n* = 7) were isolated in Colombia and belonged to the high-risk clone ST235. To note, none of these isolates showed mutations in *bla*$_{\text{KPC-2}}$.

To explore in detail the molecular mechanisms previously associated with resistance to CZA in these *P. aeruginosa* clinical isolates, we analyzed a variety of genes for any mutation that could lead to overexpression or repression of a particular gene, or to amino acid substitutions that could change the activity of the protein. These genes include β-lactamase encoding genes (e.g., *bla*$_{\text{PDC}}$) and their regulator genes (*bla*$_{\text{AmpD}}$, *bla*$_{\text{AmpR}}$, *bla*$_{\text{AmpG}}$); genes encoding the multidrug efflux MexA-B, and its regulators (MexR, NalC and NalD); (*ftsI*, and *dacB* encoding PBP3 and PBP4, respectively); *creD*, which encodes a predicted inner membrane protein part of the conserved two-component regulatory system CreBC (24); and genes involved in pathogenesis like DnaJ, DnaK, and ATP-dependent Clp protease proteins (13, 25–27).

Specifically, predicted substitutions in AmpG, DnaJ, DnaK, and ATP-dependent Clp protease proteins were not found. The proteins that had substitutions in most isolates were PDC, PoxB/OXA-50-like, NalC, and CreD. Most of the proteins had multiple substitutions, except peptidases S41, PBP3/FtsI and NalD, which had only one substitution in some isolates (Table S3). Substitutions in MexAB-OprM regulator proteins, most frequently a G71E change in NalC (77%) and a V126E substitution in MexR (47.5%) were observed. Mutations leading to substitutions in PBP3, PoxB, and the PDC/AmpC system were detected in 9.8%, 95.1%, and 82% of the *P. aeruginosa* CZA-resistant isolates, respectively. Only six isolates had the substitution N117S in PBP3, all of them belonging to the ST309 from Mexico (four), Colombia (one), and Chile (one) (Table S3).

Of special interest, clonal spread of the mutations linked to particular STs was observed in our results. In all isolates belonging to the ST235 recovered from Colombia, Argentina, and Mexico, we found identical substitutions in PDC (G1D, A71V, T79A, V179L, and G365A), AmpG (A583T), AmpR (G283E, M288R), and AmpD (G148A). Similarly, in all isolates belonging to the ST244 from Argentina, Brazil, and Colombia, identical substitutions were observed in CreD (Q253E, A394V, F445L, R451K, I469A), AmpD (G148A, D183Y), AmpG (A583T), and PoxB (L6F, R49C), compared to *P. aeruginosa* PAO1. Likewise, strains belonging to the ST309 from Mexico, Chile, and Colombia had identical substitutions in CreD (D95N, V335I, A394V, F445L, and I469A), AmpG (A583T), AmpR (G283E, M288R), MexA (K16K), MexR (V126E), and PBP3 (N117S). Lastly, isolates belonging to ST575, all isolated in Mexico, had the same substitutions in DacB (A394P), CreD (F445L, R451K, and I469A), AmpG (A583T), PDC (T79A), OprD (D43L, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296Q, and Q300E), and MexR (Δ1-4aa) (Table S3).

TABLE 1 Breakdown of ceftazidime-avibactam resistance rates among *Enterobacterales* and their associated resistance genes

Bacterial species	No. of isolates tested	Resistant isolates n (%)	Positive isolates for Carba-NP	Positive isolates qPCR (%)			
				bla_{KPC}	bla_{NDM}	bla_{KPC} + bla_{NDM}	bla_{VIM}
E. coli	1,397	1 (0.07)	1	0 (0)	1 (0.07)	0 (0)	0 (0)
K. pneumoniae	607	13 (2.1)	13	0 (0)	10 (1.6)	3 (0.5)	0 (0)
E. cloacae complex	112	3 (2.7)	3	0 (0)	1 (0.9)	1 (0.9)	1 (0.04)
S. marcescens	90	1 (1.1)	1	0 (0)	1 (1.1)	0 (0)	0 (0)
K. aerogenes	29	0	0	0 (0)	0 (0)	0 (0)	0 (0)
Total	2,235	18 (0.8)	18	0	13 (0.6)	4 (0.2)	1 (0.04)

TABLE 2 Percentage of resistance of *P. aeruginosa* to ceftazidime-avibactam and comparator agents by country[a]

Country	No. of isolates	CZA		CAZ		FEP		PTZ		IMI		MER	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Colombia	239	59	(25)	103	(43)	98	(41)	124	(52)	164	(69)	122	(51)
Chile	61	7	(11)	26	(43)	22	(36)	25	(41)	35	(57)	22	(36)
Argentina	29	10	(34.4)	19	(66)	15	(52)	20	(69)	20	(69)	17	(59)
Mexico	124	28	(23)	61	(49)	63	(51)	61	(49)	93	(75)	68	(55)
Brazil	39	5	(13)	13	(33)	22	(56)	20	(51)	34	(87)	28	(72)
Total	492	109	(22.1)	222	(45)	220	(45)	250	(51)	346	(70)	257	(52)

[a]CZA, ceftazidime avibactam; CAZ, ceftazidime; FEP, cefepime; PTZ, piperacillin tazobactam; IMI, imipenem; MER, meropenem.

TABLE 3 Breakdown of ceftazidime-avibactam resistance rates among *P. aeruginosa* and their associated resistance genes

Country	No. of isolates tested	Resistant isolates n (%)	Positive isolates for Carba-NP	Positive isolates qPCR (%)				
				bla_{KPC}	bla_{VIM}	bla_{KPC} + bla_{VIM}	bla_{IMP}	bla_{SPM-1}
Colombia	239	59 (24.7)	39	8 (3.3)	24 (10)	7 (2.9)	0 (0)	0 (0)
Chile	61	7 (11.5)	3	0 (0)	3 (4.9)	0 (0)	0 (0)	0 (0)
Argentina	29	10 (34.5)	2	0 (0)	0 (0)	0 (0)	2 (6.9)	0 (0)
Mexico	124	28 (22.6)	5	0 (0)	4 (3.2)	0 (0)	1 (0.8)	0 (0)
Brazil	39	5 (12.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.6)
Total	492	109 (22.1)	49	8 (1.62)	31 (6.3)	7 (1.4)	3 (0.6)	1 (0.2)

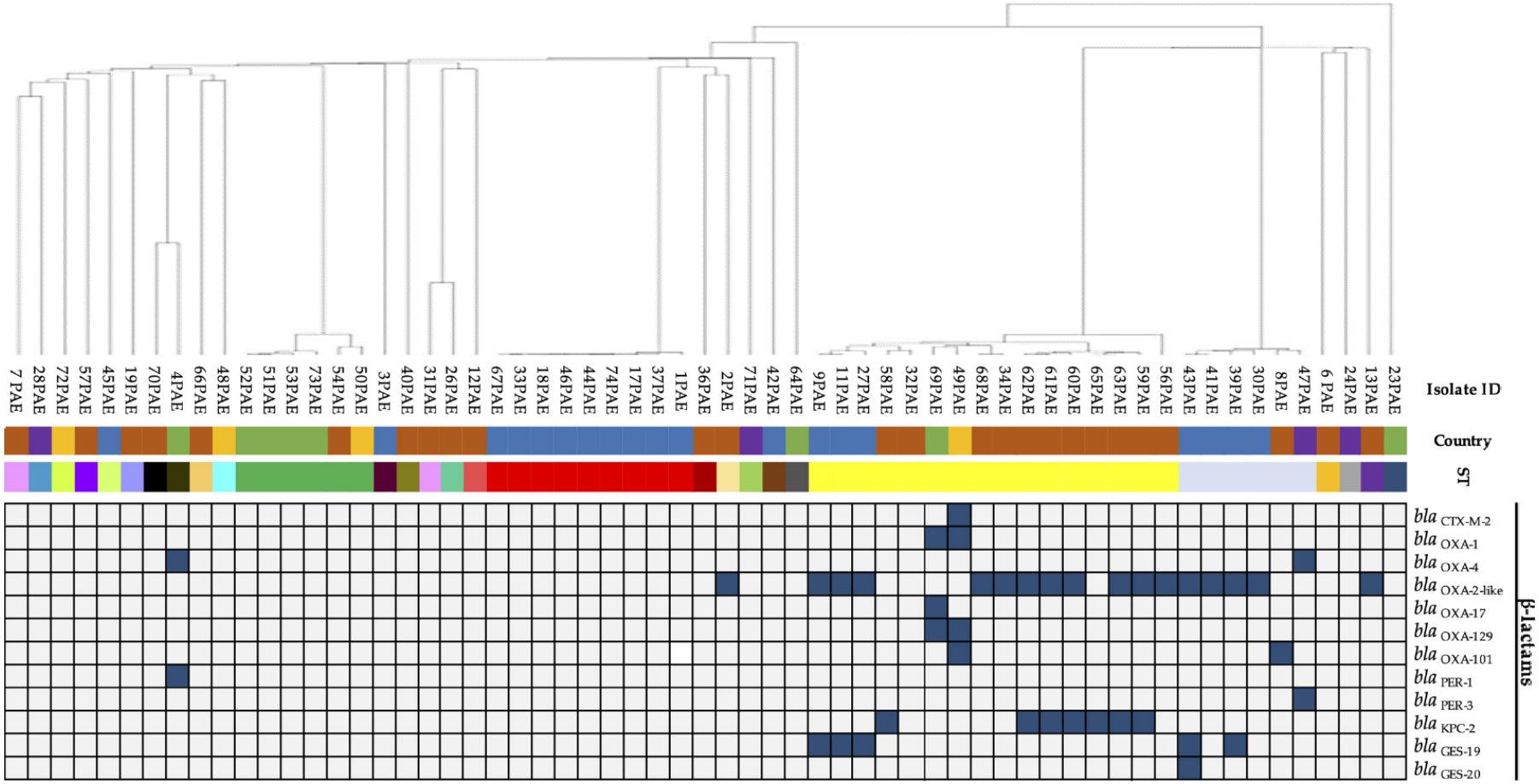

FIG 1. Genetic resistance determinants of CZA-resistant *P. aeruginosa* isolates. Blue squares represent the presence of the respective antibiotic resistance gene, and blank represents its absence. The phylogenetic tree was obtained by canonical wgMLST using the web server cano-wgMLST_BacCompare (25) and drawn using the iTOL tool (50). ST, sequence type. New ST are indicated by *.

Discussion

In a previous study, we evaluated the *in vitro* activity of CZA against a set of 2,252 clinical isolates of *Enterobacterales* in Latin America, finding that 4.2% were resistant (23). However, combined phenotypic tests performed in this study confirmed the CZA-resistant phenotype of only 18/94 isolates. Therefore, the updated resistance rate to CZA of this group of *Enterobacterales* is 0.8%. Additionally, we analyzed the susceptibility to CZA of a set of 492 clinical isolates of *P. aeruginosa* collected during the same time period (2016 to 2017) in the same five Latin American countries. Finally, we determined the molecular mechanisms leading to CZA resistance in these isolates by WGS.

Several molecular mechanisms leading to decrease susceptibility to CZA have been described in *P. aeruginosa*. Among them, specific amino acid substitutions in some β-lactamases, including KPC and SHV have been associated with resistance to CZA (11). In particular, the D179Y substitution in the Ω-loop of KPC-3, and in other KPC variants, confer resistance to CZA. Of note, this mechanism was reported in a *P. aeruginosa* isolate from Chile before this antibiotic was clinically available in this country (28). Interestingly, all sequenced *P. aeruginosa* isolates that carried bla_{KPC-2} retrieved in Colombia belonged to ST235. This ST has been associated with the disseminations of bla_{KPC-2} in Colombia (2). As we did not evidence any mutations in bla_{KPC-2}, CZA-resistance is most probably caused by other mechanisms. All of these seven strains (58PAE to 63PAE and 65PAE in Table S3) have multiple mutations in several genes, including in *ampR* leading to the substitutions G283E, M288R in AmpR, and mutated *ampG*, producing the variant A583T. The association of these mutations with CZA resistance is yet to be determined. Moreover, six out of seven isolates showed mutations in *nalD* (coding for the MexAB-oprM regulator), which could lead to decreased susceptibility to CZA as previously reported (29, 30) (Table S3).

Regarding the molecular epidemiology, WGS analysis revealed that some of the CZA-resistant *P. aeruginosa* isolates belonged to ST235 ($n = 16$), ST244 ($n = 6$), and ST111 ($n = 1$). These STs have been considered as high-risk clones (31, 32). Furthermore, ST235 and ST111 are multidrug resistant (MDR) clones disseminated worldwide and linked to the expression of VIM-2 (2). Sixteen of

the sequenced isolates belonging to ST235 did not harbor any *bla*$_{VIM}$ gene but all harbored *bla*$_{KPC}$. A surveillance study of *P. aeruginosa* performed in Colombia found that ST111 is a common host of *bla*$_{VIM-2}$, whereas ST235 is associated with *bla*$_{KPC-2}$, as aforementioned (33). Additionally, an isolate that carried *bla*$_{SPM-1}$ belonged to ST277, which is a ST commonly associated with the dissemination of *bla*$_{SPM-1}$ in Brazil (12).

Extended-spectrum β-lactamases (ESBL) such as PER and GES have also been associated with resistance to CZA via biochemical mechanism conferring a weaker inhibitory potency of avibactam to these enzymes (34). This kinetic feature, possibly combined with the lower permeability of *P. aeruginosa*, can effectively decreased the susceptibility to CZA (9, 13, 34, 35). In our study, one *P. aeruginosa* isolate from Argentina (ST179) and one isolate from Chile (ST309) harbored *bla*$_{PER-1}$ and *bla*$_{PER-3}$, respectively. In addition, five isolates from Mexico carried *bla*$_{GES-19}$, three of them were ST235 and the other two were ST309. In Mexico, a high prevalence of the ESBL GES-19 and the carbapenemase GES-20 have been reported as the most prevalent in *P. aeruginosa* (36). Moreover, it has been reported that the presence of the ESBL-encoding genes *bla*$_{GES-19}$ and *bla*$_{GES-26}$ in tandem is associated with resistance to all β-lactams, including CZA (21). Importantly, in the present study one of the *P. aeruginosa* isolates belonging to ST309 showed a similar feature, where *bla*$_{GES-19}$ and *bla*$_{GES-20}$ were found in tandem, which might explain the resistance to CZA. Dissemination of *P. aeruginosa* isolates harboring either *bla*$_{PER}$ or *bla*$_{GES}$ genes is worrisome, as production of these enzymes compromise the efficacy of the latest anti-pseudomonal drugs, CZA and ceftolozane-tazobactam (14, 37).

A recent study by Fraile-Ribot et al. found that the duplication of the residue D149 in OXA-2 led to resistance to CZA *in vivo* (8). This new variant of OXA-2, called OXA-539 was reported for the first time in a *P. aeruginosa* isolate resistant to CZA belonging to ST235, from a patient with a susceptible isolate who was previously treated with CZA (8). In our analysis, 17 *P. aeruginosa* isolates carried OXA-2, 11 of them belonging to ST235 but none of them had the D149 duplication. Worth noting, all *P. aeruginosa* resistant to CZA and harboring *bla*$_{OXA-2}$ were exclusively recovered from Mexico and Colombia.

Several enzymes of class D, including PoxB (OXA-50-*like*), which is encoded in the chromosome of all *P. aeruginosa* strains, are not efficiently inhibited by DBOs (38). Compared to the PoxB encoded in the reference strain *P. aeruginosa* PAO1, multiple substitutions in PoxB were found in our isolates. However, there is no evidence that these mutations can lead to resistance to CZA. On the contrary, Castanheira et al. described substitutions in PoxB in both susceptible and resistant isolates, suggesting that these changes are not directly leading to CZA-resistance (25).

Although CZA shows potent inhibitory activity against PDC (AmpC) of *P. aeruginosa*, mutations in bla_{PDC} conferring resistance to CZA have been reported (39). Here, we found 14 different PDC variants, being PDC-3, PDC-35, and PDC-1 the most frequent (Table S3). However, these variants have not been associated with a particular antimicrobial resistance pattern in previous studies. Moreover, previous investigations have suggested that amino acid substitutions in the PDC enzyme are unlikely to be the main mechanism conferring resistance to CZA, because a correlation between the PDC enzyme variations and the MIC has not been detected (40). However, the recent emergence of *P. aeruginosa* clinical isolates overexpressing variants of PDC is worrisome and may compromise the efficacy of CZA (40). Indeed, the E247K, G183D, T96I, and ΔG229 to E247 substitutions and deletions appear to perform a 2-fold effect on the catalytic cycle of PDC, allowing to evade avibactam inhibition, while hydrolyzing ceftazidime with enhanced efficiency (40). More biochemical studies are needed to elucidate the relation between the PDC variants identified in this study and CZA-resistance in *P. aeruginosa*.

As previously mentioned, changes in PBPs can lead to CZA resistance. For instance, FtsI (PBP3) of *P. aeruginosa*, is the PBP to which many β-lactams, including monobactams and some cephalosporins, have the highest affinity for. FtsI is the primary target of ceftazidime, however, avibactam is also known to covalently bind to the PBPs of *P. aeruginosa* (1). The FtsI variants R504C and P527S have been strongly associated with reduced susceptibility to different types of β-lactams, including ceftazidime (5). We did not find these mutations in our isolates. However, six sequenced *P. aeruginosa* isolates showed the same FtsI variant, N117S, which, has not been associated to CZA resistance, and

given its location within the protein, an effect on CZA-resistance is unlikely. Interestingly, all the strains harboring the N17S variant of FtsI belonged to the ST309, which has been described in serious infections involving MDR and XDR *P. aeruginosa* strains. Furthermore, all six isolates were recovered from different geographical locations Mexico, Colombia, and Chile, suggesting that the geographic distribution of ST309 is widespread (21).

A study from Castanheira et al. showed that MexAB-OprM efflux system overexpression was significantly associated with CZA resistance, alone or in combination with alterations or disruptions in other genes (25). Furthermore, it has been shown that disruption of MexR, a negative regulator of MexAB-OprM, leads to high expression of the MexAB-OprM efflux pump slightly raising the MIC of CZA (41). In our analysis, nine isolates belonging to the ST575 from Mexico showed altered versions of MexR. Additionally, 18 isolates (5 from Argentina [ST244 and ST179], 11 from Colombia [ST235 and ST3963], 1 from Chile [ST357], and 1 from Brazil [ST235]) had mutations, framework-shifts, or alterations in the NalD, a repressor of MexAB. Mutations in NalD have been associated with hyperexpression of MexAB and therefore, resistance of all β-lactams (30).

Regarding the *Enterobacterales*, we determined that the presence of at least one MBL-encoding gene in all evaluated isolates could be the underlying molecular mechanism leading to CZA-resistance. The presence of MBL-encoding genes in CZA-resistant *Enterobacterales* has been frequently reported in the United States, countries of the Asian-Pacific region, and Europe (9, 42, 43).

Interestingly, all CZA-resistant *Enterobacterales* were isolated in Colombia, where KPC-enzymes are considered endemic (44). Although specific amino acid substitutions in the Ω-loop of KPC leading to CZA-resistance in *Enterobacterales* have been reported in several countries, we did not find isolates harboring *bla*$_{KPC}$ without an MBL-encoding gene. Conversely, the prevalence of *Enterobacterales* carrying MBL, especially NDM, either alone or in combination with a serine carbapenemase has increased in recent years in

this country (45). Exemplary for this observation, we found four isolates from Colombia harboring both *bla*$_{KPC}$ and *bla*$_{NDM}$ (1, 10, 42).

Conclusions

By the time of the collection of these isolates, a low rate of resistance to CZA was found among *Enterobacterales* in the Latin American countries that participated in this study. In this analysis, we demonstrated that the most common mechanism of resistance in *Enterobacterales* was the production of MBLs. In contrast, resistance to CZA in *P. aeruginosa* has proven to be more complex, as it might involve multiple known and possibly unknown resistance mechanisms.

Our study has many limitations. Due to budget restrictions, we could only sequence some of the CZA-resistant isolates and none of the CZA-susceptible ones. This impeded us to have the complete molecular snapshot of all *Enterobacterales* and *P. aeruginosa* isolates. Consequently, we are only reporting known mechanisms of reduced susceptibility to CZA in these isolates. More studies are needed to investigate emerging mechanisms of resistance to CZA. Nevertheless, as these isolates were collected before the clinical use of CZA in Latin America, the results presented here offer a valuable tool for upcoming comparisons with isolates of *Enterobacterales* and *P. aeruginosa* recovered after its introduction in this region. These studies will delineate the evolutionary path of the CZA-resistance and how its use in the clinical practice affects the epidemiology of these MDR pathogens. The knowledge of the evolution of resistance to last-resort antibiotics such as CZA in clinical isolates will help to understand the role of selective pressure in different scenarios.

Ethical approval

The protocol was approved by the ethics committee of Universidad El Bosque, under act #018-2020. Collection of the microbiological isolates was part of the regular diagnostic process, as established by each of the participating health care institutions.

Materials and methods

Susceptibility testing and detection of carbapenemases

Resistance to CZA was confirmed by MICs determined by broth microdilution method using customized Sensititer plates (Trek Diagnostic Systems, Thermo Fisher Scientific, UK) following the manufacturer's recommendations and, Etest (bioMérieux, Marcy l'Etoile, France). Results were interpreted according to the current guidelines of the Clinical and Laboratory Standards Institute (CLSI) (46). Presence of carbapenemases in CZA-resistant *Enterobacterales* and *P. aeruginosa* isolates was initially screened by RAPIDEC Carba-NP Assay (bioMérieux, Marcy-l'Étoile, France) (47), followed by qPCR targeted to the bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, $bla_{oxa\text{-}48\text{-}like}$, and $bla_{SPM\text{-}1}$ genes. The reference strains *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were used as the quality control strain, as per CLSI recommendations (46).

Whole-genome sequencing

Genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic sequencing of 67 clinical isolates *of P. aeruginosa* were performed on the Illumina MiSeq platform (Illumina Inc., San Diego, California, USA) with 250 nt paired end reads to achieve a coverage of about 30× per base, using MiSeq V3 flow cell. *de novo* assembly were performed using CLC Genomics Workbench 8.1.0 software and annotation was done by using RAST server. Multi locus sequence type (MLST) 1.8 server was used to determine the sequence type (ST) of *P. aeruginosa* isolates (https://cge.food.dtu.dk/services/MLST/) (48). Additionally, antibiotic resistance genes were predicted using online databases (https://cge.food.dtu.dk/services/ResFinderFG/) (49). The genome of *P. aeruginosa* PAO1 (GenBank ID: NC_002516.2) was used as reference, in order to look for known alterations and disruptions in proteins involved in efflux, regulation of PDC, PBPs, and others associated with CZA resistance. The proteins analyzed were PDC (AmpC), AmpR, AmpG, AmpD, FtsL (PBP-3),

PoxB (OXA-50-like), DacB (PBP-4), CredD, MexA, MexB, MexR, OprD, DnaJ, DnaKATP-dependent Clp protease proteins, NalC and NalD (25).

The analyses of the 61 CZA-resistant *P. aeruginosa* isolates were conducted using cano-wgMLST_BacCompare web-based tool (http://baccompare.imst.nsysu.edu.tw) (50), while the cano-wgMLST tree was built using the highly discriminatory loci among isolates. The dendrogram was visualized with iTOL v6 (http://itol.embl.de) (51).

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Darstellung des eigenen Anteils

In vitro susceptibility to ceftazidime/avibactam and comparators in clinical isolates of Enterobacterales from five Latin American countries

- Analyse der Daten
- Schreiben des Manuskripts

Evaluation of in vitro susceptibility to ceftazidime/avibactam of clinical isolates of carbapenem non-susceptible gram-negative bacilli from Colombia

- Empfindlichkeitstestung der *P. aeruginosa* Isolate
- PCR der CZA-resistenten Isolate
- Erstellen und Präsentation des Posters

Molecular mechanisms of resistance to ceftazidime/avibactam in clinical isolates of Enterobacterales and *Pseudomonas aeruginosa* in Latin American hospitals

- Empfindlichkeitstestung
- Analyse der Daten
- Überarbeitung des Manuskripts

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Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Arbeit mit dem Titel

Empfindlichkeit und molekulare Resistenzmechanismen gegenüber Ceftazidim/Avibactam in gramnegativen Mikroorganismen aus fünf lateinamerikanischen Ländern

im Zentrum der Inneren Medizin, Medizinische Klinik 2 unter Betreuung und Anleitung von Prof. Dr. med. Maria Vehreschild ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Vorliegende Ergebnisse der Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

- Mojica MF, De La Cadena E, García-Betancur JC, Porras J, Novoa-Caicedo I, Páez-Zamora L, Pallares C, Appel TM, Radice MA, Castañeda-Méndez P, Gales AC, Munita JM, Villegas MV. Molecular Mechanisms of Resistance to Ceftazidime/Avibactam in Clinical Isolates of *Enterobacterales* and *Pseudomonas aeruginosa* in Latin American Hospitals. mSphere. 2023 Mar 6:e0065122.
- Appel TM, Mojica MF, De La Cadena E, Pallares CJ, Radice MA, Castañeda-Méndez P, Jaime-Villalón DA, Gales AC, Munita JM, Villegas MV. In vitro susceptibility to ceftazidime/avibactam and

comparators in clinical isolates of *Enterobacterales* from five Latin American countries. Antibiotics (Basel) 2020; 9(2):62

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