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Evaluation of different therapeutic options against extensively drug-resistant (XDR) *Pseudomonas aeruginosa*, including high-risk clones, using different *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) models

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Certificamos,

Que la Tesis Doctoral titulada **"Evaluation of different therapeutic options against extensively drug-resistant (XDR) *Pseudomonas aeruginosa*, including high-risk clones, using different *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) models"**, presentada por **Sandra Domene Ochoa** y dirigida por nosotros, representa una aportación relevante al tema y reúne méritos suficientes para ser presentada y defendida ante el Tribunal correspondiente para optar al Grado de **Doctor en Microbiología**, del departamento de Genética y Microbiología-Universitat Autònoma de Barcelona.

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Barcelona, 12 de enero de 2023.

Acknowledgements

Porque todos los finales sean el inicio de nuevas oportunidades,
desafíos y sueños por cumplir.

A Mila, por ser soporte y empuje. Por lo mucho que he aprendido a tu lado y tu confianza en mí. Por todo lo recorrido y sobre todo por lo que vendrá, siempre juntas a la par. Por seguir compartiendo entre ciencia, las metas, confesiones, kilómetros, consejos, alegrías y proyectos. Gracias eternas… ¡¡Seguimos!!

A Juan Pablo, por las oportunidades
brindadas. Por el constante apoyo y dedicación.

Als papis, per donar-me arrels i ales. Per la paciència i per permetre'm arribar fins aquí. A l'Alex, la meva sort, per fer-me la vida més fàcil farà ja 30 anys.

Al Bruno, pel suport incondicional.

A la Berta, per convertir-se en companya de feina i de vida. Perquè entre el miracle i el desastre, segueix sent més fàcil al teu costat.

A los amigos que hacen más fáciles las subidas para disfrutar de las bajadas.

A las que me complementan y completan: Maria, Ari, Adriana: porque, aunque a veces apriete y ahogue, son familia y hogar.

Al Servicio de Enfermedades Infecciosas del Hospital del Mar por la formación y por hacer posible este proyecto. A Inma, Dani, Luisa y Hernando, por su soporte durante este tiempo. A Marisa y Tere por su alegría.

A Carme por ser tan buena. A Silvia por su compañía.

A la Núria, per ensenyar-me que la feina feta mai fa destorb i per transmetre'm coneixements i formació. A Eduardo, por su ayuda y generosidad.

Al Ferran, per la seva ajuda i disponibilitat.

Al Laboratorio de Referencia de Catalunya, por brindarme las herramientas necesarias para el desarrollo del presente trabajo.

Al equipo de Mallorca, por su indispensable ayuda.

Acronyms & Abbreviations

CFU:	Colony forming units
cIAI:	Complicated intra-abdominal infection
CI:	Continuous infusion
CMS:	Colistimethate sodium
Css:	Steady-state concentration
C/T:	Ceftolozane/Tazobactam
cUTI:	Complicated urinary tract infection
CZA:	Ceftazidime/Avibactam
DTR:	Difficult-to-treat resistance
EMA:	European Medicines Agency
ESBL:	Extended-spectrum beta-lactamase
FDA:	Food and drug administration
h:	Hour
HABP:	Hospital-acquired bacterial pneumonia
HAI:	Healthcare-associated infection
LPS:	Lipopolysaccharide lipid A
MBL:	Metallo-beta-lactamase
MDR:	Multidrug-resistant
MIC:	Minimal inhibitory concentration
PBP:	Penicillin-binding protein
PD:	Pharmacodynamic
PDR:	Pandrug-resistant
PK:	Pharmacokinetic
QRDR:	Quinolone Region Drug Resistance
ST:	Sequence Type
T>MIC:	Time above MIC
VAPB:	Ventilator-associated bacterial pneumonia
XDR:	Extensively drug-resistant

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1. ABSTRACT

The indiscriminate use of antibiotics has contributed to the emergence and spread of extensively drug-resistant (XDR) *Pseudomonas aeruginosa.* The increase in XDR strains seriously compromises antibiotic treatment options and led to higher morbidity and mortality rates among patients with *P. aeruginosa* infections. New therapeutic options are required to overcome the growing problem of antimicrobial resistance compounded by a dwindling supply of new drugs.

The main objective of this thesis is to evaluate different therapeutic options against extensively drug-resistant (XDR) *P. aeruginosa*, including high-risk clones, using different *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) models.

In the first part of the studies, different antibiotic combinations have been evaluated: colistin plus meropenem, ceftolozane/tazobactam (C/T) plus colistin and ceftazidime/avibactam (CZA) plus colistin, amikacin and aztreonam. Antipseudomonal combination therapy led to increased activity against XDR *P. aeruginosa* compared with that of either agent used as a monotherapy, and could prevent resistance development. Combination therapy would benefit patients with severe *P. aeruginosa* infections.

Secondly, it was intended to optimize PK/PD antimicrobial properties when treating XDR *P. aeruginosa* infections. Since C/T it is a time-dependent antimicrobial, the actual standard dose could be optimized when dealing with XDR *P. aeruginosa.* C/T in continuous infusion (CI) achieved a greater overall reduction in bacterial burden than intermittent or extended dosing regimens. CI regimen has demonstrated to be a useful strategy, but it would be necessary to adjust antibiotic steady-state concentration (Css). The administration of suboptimal Css resulted in the emergence of C/T resistance, whereas higher Css showed a slight advantage in effectiveness.

These *in vitro* observations provide promising data that are of value as a basis for expanding antibiotic research and ultimate evaluation in clinical use. Findings may help to identify novel strategies to improve the treatment of XDR *P. aeruginosa* infections.

2. INTRODUCTION

Antibiotic resistance has been existing since ancient times (1) but nowadays it has become a serious worldwide health problem associated to more than 0.7 million deaths per year (2,3), currently being considered one of the largest health threats (4). Bacteria can easily acquire new antibiotic resistance through chromosomal mutations and horizontal gene transfer (5). The indiscriminate use of antibiotics in clinic or agriculture has highly contributed to the emergence and selection of antibiotic multidrug-resistant and extensively drug-resistant pathogens (MDR and XDR, respectively) (2) causing a shortage of antibiotic therapeutic alternatives that hinder the choice of the proper treatment (4) and, consequently, morbidity and mortality rates have increased notably.

Currently, the classification of bacterial isolates with some type of resistance is carried out based on the criteria established by Magiorakos et al (6). The strains are considered MDR when they are non-susceptible (intermediate plus resistant) to at least three antibiotics classes; XDR when they are non-susceptible to all antibiotics categories with the exception of two; and finally, pan-drug resistant (PDR), which are those resistant to all antibiotics categories (6). More recently, difficult-to-treat resistance (DTR) was introduced in Gram-negative bacteria. DTR signifies no active first-line agents and represents an even higher level of resistance (7).

Healthcare-associated infections (HAIs) include the classical nosocomial infection (i.e., those acquired in the hospital), and infections in patients routinely attended by day hospital services and those from long-term care facilities. These HAIs may be more difficult to treat and may have a worse clinical outcome when caused by MDR/XDR bacteria. Among these bacteria, the ESKAPE group includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (6,8). These microorganisms have often been involved in nosocomial outbreaks (1,9).

One of the most important microorganisms with an outstanding capacity to develop resistance is *P. aeruginosa* (10). *P. aeruginosa* is a Gram-negative, non-fermenting and rod-shaped bacterium. It is a microorganism with a wide distribution with simple nutritional requirements and great metabolic versatility. Although considered strict aerobic, some strains can grow slowly in an anaerobic environment with the presence of nitrate. Despite not being commonly present in normal human microbiota, it may transiently colonize human. It is a causal agent of opportunistic infections, being one of the most relevant nosocomial pathogens in our environment (11). Transmission in hospitals may be from environmental sources (especially humid reservoirs, such as respiratory equipment, nebulizers and showers) or human-mediated (including sanitary personnel) (12,13).

Among the predisposing clinical factors of these infections are immunosuppression, the use of medical devices (such as mechanical ventilation or urinary catheters), and previous antibiotic treatment (14–16). *P. aeruginosa* is involved in respiratory tract infections (such as cystic fibrosis or chronic obstructive pulmonary disease, as well as in ventilator associated pneumonia in critically ill patients) (10). This bacterium is also related to bacteraemia, urinary infections and a range of soft tissue infections, including folliculitis, external otitis and infections of large burns (5).

P. aeruginosa produces a wide range of virulence factors that contribute to colonization and pathogenesis, including adhesins (pili, flagella), secretion systems (Type I, II and III), hydrolytic enzymes and siderophores. One of the most important virulence determinants is the type III secretion system (TTSS) which injects effector cytotoxins into the host cells. One of this cytotoxins (ExoU) is the most potent identified, and its expression correlates with a poor prognosis (17). This bacterium is able to form biofilm through alginate production, which plays an important role in the pathogenesis (18,19).

In order to define each *P. aeruginosa* profile, the following classes and antibiotics were recommended for testing: antipseudomonal cephalosporins (ceftazidime and cefepime), antipseudomonal penicillin plus beta-lactamase inhibitors (ticarcillin-

clavulanate and piperacillin-tazobactam), monobactams (aztreonam), antipseudomonal carbapenems (imipenem, meropenem and doripenem), aminoglycosides (gentamicin, tobramycin, amikacin and netilmicin), fluoroquinolones (ciprofloxacin and levofloxacin), phosphonic acids (Fosfomycin) and polymyxins (colistin and polymyxin B) (17).

2.1. *Pseudomonas aeruginosa* resistance mechanisms

Antibiotic resistance in *P. aeruginosa* can be caused by numerous mechanisms. *P. aeruginosa* presents intrinsic resistance to a wide range of antibiotics (12). Apart from its vast intrinsic resistome, *P. aeruginosa* has the ability to acquire resistance easily (12). Adaptive resistance can also occur, due to changes in gene expression as a consequence of environmental stimuli (including exposure to antibiotics such as colistin) (16). When the triggering stimulus disappears, the bacteria revert to the initial responsive phenotype. *P. aeruginosa* usually presents several simultaneous resistance mechanisms, making it difficult to infer the resistance mechanism based on the phenotypic resistance pattern (20).

2.1.1. Intrinsic resistance (intrinsic resistome)

The intrinsic resistance mechanisms are mainly due to the low permeability of its outer membrane, the constitutive and inducible expression of expulsion pumps (efflux-pumps) and the expression of chromosomal enzymes (i.e. inducible AmpC cephalosporinase expression) (17,21). Inducible beta-lactamase production has a key role in the natural resistance to aminopenicillins and some first and second class of cephalosporins. These beta-lactams antibiotics are strong inducers of beta-lactamase expression and are efficiently hydrolysed by AmpC. Also, it plays a role in the natural reduced susceptibility to imipenem (17). Constitutive expression of MexAB-OprM efflux pump leads to lower basal levels of susceptibility to the majority of beta-lactams (except for imipenem), fluoroquinolones, macrolides, cotrimoxazole, tetracyclines, chloramphenicol and sulphonamides. Inducible expression of MexXY plays a role in lower basal levels of susceptibility to aminoglycosides (17,22). Moreover, there are a large set of genes referred to

P. aeruginosa intrinsic resistome that have an effect on antibiotic susceptibility (16,23–25).

2.1.2. Acquired resistance

In *P. aeruginosa*, acquired resistance is mainly mutational (endogenous). The acquisition of foreign resistance genes (beta-lactamases and aminoglycoside-modifying enzymes) through horizontal gene transfer (through plasmids, transposons, integrons and prophages) has been described with increasing frequency, and constitutes a risk due to its ability to spread. These mutations can change an antibiotic target or the expression of an intrinsic resistance mechanism (5,8).

- **Acquisition of resistance through chromosomal gene mutations (mutational resistome)**

In *P. aeruginosa*, resistance due to chromosomal mutations is related to a hyperproduction of the chromosomal AmpC beta-lactamase, porin OprD repression and hyperexpression of expulsion pumps. In wild-type strains, the expression of the ampC gene is repressed by the *ampD* regulatory gene. Mutations in this regulatory gene can cause derepression of the *ampC* gene and lead to hyperproduction of AmpC (26). The hyperproduction of this beta-lactamase determines resistance to antipseudomonic penicillins (including combinations with inhibitors), cephalosporins, and monobactams. Apart from AmpC hyperproduction, mutations leading to structural modification of AmpC may cause beta-lactams resistance, including the novel beta-lactam-beta-lactamase inhibitor combinations ceftolozane-tazobactam (C/T) and ceftazidime-avibactam (CZA) (17).

P. aeruginosa has four main porins (OprF, OprC, OprE and OprD). Loss or reduction of its expression determines less access of antibiotics to its target. While most beta-lactams get accessed by the OprF porin, some carbapenems can be accessed by the OprD porin (but not other classes of beta-lactams). Mutations leading to loss or deceased expression in OprD porin are associated with

resistance to imipenem and reduced susceptibility to meropenem (26–28). OprD inactivation frequently acts synergistically with AmpC hyperexpression resulting in resistance to all the classis antipseudomonal beta-lactams (17,29).

P. aeruginosa has systems for the active expulsion of toxic substances, including antimicrobials. In this microorganism the identified systems are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (30). MexAB-OprM is expressed constitutively. The hyperproduction of this system is caused by mutations that affect the regulation of its expression (*mexR* regulatory gene) and is associated with resistance to carbapenems (especially meropenem), other beta-lactam antibiotics alone or in combination with beta-lactamase inhibitors (with the exception of imipenem) and fluoroquinolones, among other antibiotics (27). Mutation-driven overexpression of MexXY results in resistance to cefepime (17). Mutations that drive MexCD-OprJ overexpression results in increased cefepime minimal inhibitory concentration (MIC) and increased susceptibility to several beta-lactams and aminoglycosides (17,31). Lastly, affecting fluoroquinolones and imipenem, MexEF-OprN hyperexpression can occur (17).

Fluoroquinolone resistance in *P. aeruginosa* is produced by different mechanisms, such as decreased permeability, active expulsion systems, and mutations in the region of determinants of resistance to quinolones (Quinolone Region Drug Resistance: QRDR). Mutations in the genes that encode the subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) cause target modification (30).

Polymyxins (colistin) increase the permeability of the outer membrane by binding to lipopolysaccharide lipid A (LPS) from Gram-negative bacteria. Polymyxin resistance can be acquired or adaptive (through prior exposure). LPS modification results in less affinity for polymyxins. Polymyxin resistance is regulated by several two-component systems, including PhoPQ, PmrAB, ParRS, CprRS, and ColRS. These systems induce *arn* operon transcription, which will produce LPS modification (32,33).

- **Horizontally acquired resistance mechanisms (horizontally acquired resistome)**

Apart from its mutational resistome, *P. aeruginosa* has the capacity to develop further resistance to other antibiotics via horizontally acquired resistance. It involves the acquisition of resistance gene or mutation in DNA elements, including plasmids, transposons, integrons, prophages and resistance islands and can be acquired by conjugation, transformation or transduction. Plasmids can contain multiple resistance cassettes leading to multidrug resistance (17).

Transferable beta-lactamases, the extended-spectrum beta-lactamases (ESBLs) and carbapenemases, are increasing concern. Genes encoding this beta-lactamases, along with determinants of aminoglycoside resistance, are found in class 1 integrons. Different types of ESBL have been described in *P. aeruginosa*, among which are the most frequently reported those in class D (OXA-2 or OXA-10) and class A (PER, VEB, GES, BEL, PME). Carbapenemases can present different molecular characteristics that are reflected in the Ambler classification (34). The most frequently reported carbapenemases in *P. aeruginosa* include class B carbapenemases and metallo-beta-lactamases (MBLs). MBLs are the most prevalent in *P. aeruginosa*, being VIM and IMP types the most frequent and widespread (27). Within class A carbapenemases, GES and KPC enzymes are the predominant (27). Carbapenemases have activity spectrum over all beta-lactams (with the exception of aztreonam). About the novel combinations, neither C/T nor CZA shows activity against MBL-producing strains. CZA show activity against class A carbapenemases. Mutations in acquired OXA beta-lactamases may lead to the emergence of resistance to both agents (4,35,36).

The most important mechanisms involved in aminoglycosides resistance are its inactivation by modifying enzymes, alterations in permeability and elimination by expulsion pumps. In *P. aeruginosa*, genes encoding aminoglycoside-modifying enzymes are acquired by horizontal transfer. The most frequent enzymes are nucleotidyltranferases (ANT(2'')-I), which confers resistance to gentamicin, tobramycin, and kanamycin; acetyltransferases AAC(3'), whose substrate is

gentamicin and AAC(6') which confers resistance to tobramycin; and phosphotransferases (which provide resistance to kanamycin, neomycin, and gentamicin) (30,37). 16S rRNA methyltransferases (such as Rmt or Arm) confer resistance to all aminoglycoside on the market, including the novel plazomicin (17). Transferable fluoroquinolone resistance driven by Qnr determinants has occasionally been detected (38).

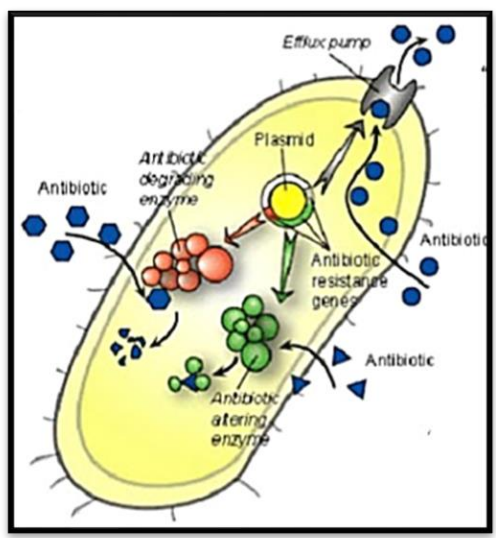

Figure 1: Main beta-lactams resistance mechanisms in *Pseudomonas aeruginosa* (27).

2.2. High-risk clones

P. aeruginosa has a non-clonal epidemic population structure, composed of a limited number of generalized clones. However, there are MDR/XDR global clones, referred to as "high-risk" clones, scattered throughout hospitals around the world (10,39). This global spread has become a public health problem due to limited therapeutic options and therapeutic costs (40). The most predominant *P. aeruginosa* high-risk clones are the sequence types (ST) ST175, ST111 and ST235 followed by ST 244 and ST 395 (10,41,42) (Figure 2) (10). Frequently related to nosocomial infections, these clones are associated with both the acquisition of horizontally transferable beta-lactamases (including carbapenemases) and chromosomal mutations (10). Of these, the ST175 clone is

widely distributed in several European countries, although outside of Europe it has only been described so far in Japan (10). Is the most common clone in clinical MDR/XDR isolates in Spain and only susceptible to colistin and amikacin. Generally, ST175 is characterized by an XDR phenotype, with resistance mainly due to mutations in QRDRs and other mutations leading to the inactivation of the OprD porin, the beta-lactamase AmpC hyperproduction and efflux-pumps overexpression (MexXY) (Figure 3) (43). It has also been related to the acquisition of an integron carrying the *aadB* gene that encodes an aminoglycoside-modifying enzyme (44).

The ST111 clone has been detected on all continents except Oceania (10). Regarding this clone, various types of ESBLs and carbapenemases have been detected, with VIM-2 being the most prevalent (10).

The ST235 clone is the one with the largest distribution worldwide (10). Although various types of acquired beta-lactamases have been detected, class B carbapenemases are the most frequent in this isolate. These include multiple IMP and VIM variants, with VIM-2 being the most prevalent (10).

The pathogenicity of epidemic high-risk clones is another major issue that should be taken into account (17,45). Considering virulence as the capacity to produce more severe infections and higher mortality in acute infections, it differs among different high-risk clones. While the virulence of ST175 seems to be particularly low, ST235 high-risk clone is highly virulent. The three major high-risk clones were found to be defective in the three types of motilities and pigment (pyoverdine and pyocyanin) production and also showed reduced fitness *in vitro*. Moreover, they displayed increased spontaneous mutant frequencies and biofilm growth (17).

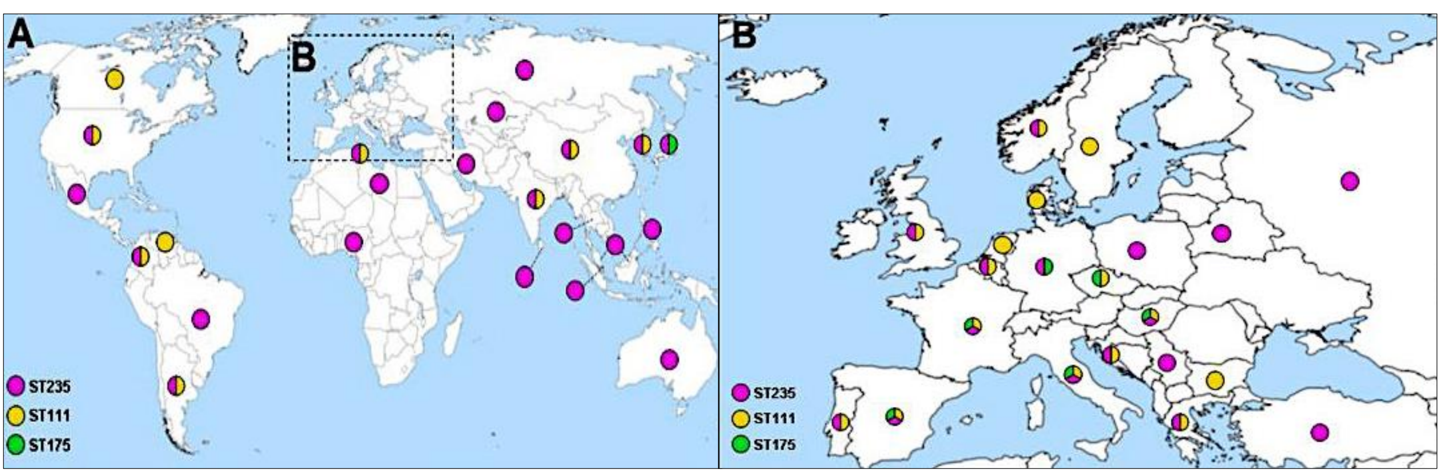

Figure 2: Distribution of *Pseudomonas aeruginosa* high-risk clones (ST235, ST111 and ST175). **A**: Worldwide distribution. **B**: Europe Distribution (10).

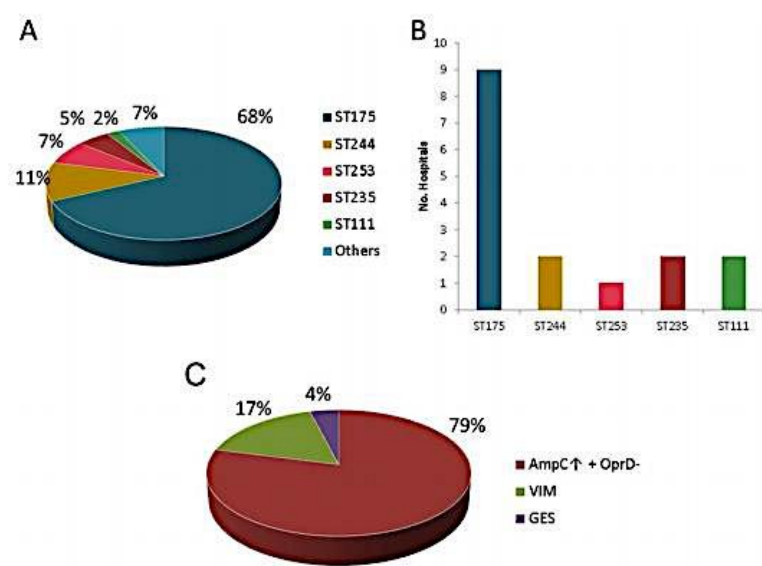

Figure 3: Distribution and resistance mechanisms in Spanish XDR *Pseudomonas aeruginosa* isolates. **A:** different high-risk clones found in Spain. **B:** Number of hospitals where the different isolates were found. **C:** beta-lactam resistance mechanism detected (43).

2.3. Therapeutic options

Useful antibiotics against *P. aeruginosa* infections are some beta-lactam antibiotics (such as piperacillin, piperacillin-tazobactam ceftazidime, cefepime,

aztreonam, imipenem, and meropenem), fluoroquinolones, aminoglycosides, fosfomycin, and polymyxins (colistin and polymyxin B). Colistin is considered a last-line antibiotic that acts at the level of the bacterial membrane interacting with its phospholipids. Polymyxins are positively charged, enabling them to interact with the phosphate groups in lipid A of the LPS that are negatively charged, causing the disruption of the outer cell membrane of most Gram-negative bacteria (46). Although it has been reintroduced in the last decade as an MDR / XDR Gram-negative bacteria treatment, its clinical use is hampered by side effects (especially nephrotoxicity), and by difficulties in establishing optimal doses (47–49). In order to reduce its toxicity, colistin was mixed with formaldehyde and sodium bisulfite, giving colistimethate sodium (CMS), which is currently administered by inhaled or intravenous form. CMS is spontaneously hydrolyzed in an aqueous medium resulting in sulfomethylated derivatives and colistin-base, which is again the active form (50,51). Polymyxin monotherapy may result in treatment failure (as reliably effective plasma exposure is not always attained), and bacterial resistance may emerge (52). Data from PK studies confirm that colistin plasma concentrations following the dosing suggestions of the European Medicines Agency (EMA) and Food and drug administration (FDA) are low and inadequate for the treatment of MDR/XDR *P. aeruginosa* infections. These findings highlight the importance of considering colistin combination therapy for MDR/XDR *P. aeruginosa* infections (17), which could broaden the spectrum of coverage, achieve and additive or synergistic antibacterial effect, and suppress emerging resistance (53–55). Among the possible combinations, colistin has been reported in combination with several antibiotics against MDR/XDR bacteria, including *P. aeruginosa* (56).

In the context of growing prevalence of MDR/XDR *P. aeruginosa* isolates showing resistance to all first-line agents, new molecules with antipseudomonic action have been developed, as well as new associations with beta-lactamase inhibitors: C/T and CZA.

Ceftazidime combined with avibactam (a new beta-lactamase inhibitor) shows an improvement in activity against beta-lactamases belonging to class A and C, as well as some enzymes of class D, but is not active against MBL-producers (57).

The addition of avibactam to ceftazidime protects the cephalosporin from enzymatic degradation caused by *P. aeruginosa* strains (mainly due to AmpC enzymes but also ESBLs and class A carbapenemases) and leads to decreased minimum inhibitory concentrations (MICs) of ceftazidime (58).

The current recommended dosage of CZA is a dose of 2/0.5 g every 8 h as a 2 h rate of infusion, for adult patients with normal renal function. As a time-dependent antibiotic, the percentage of free drug concentration that remains above the MIC for the 40-70% of the dosing interval is the best PK parameter. It is maximized when concentrations in plasma are 4-5 x MIC (59,60).

C/T arises as a new potential agent to treat XDR *P. aeruginosa* infections. C/T combines ceftolozane, a novel oxyimino-aminothiazolyl cephalosporin that has bactericidal action with tazobactam, a sulfone beta-lactamase inhibitor in fixed 2:1 ratio producing a synergistic effect (61). Ceftolozane has a side chain that confer less susceptibility to hydrolysis by the derepressed chromosomal beta-lactamase AmpC producing *P. aeruginosa*, and its effectiveness is not affected by efflux pump expression or changes in porin permeability (such as deletion of the membrane protein OprD). In order to carry its function, C/T binds to important penicillin-binding proteins (PBP), AmpC enzymes and other beta-lactamases such as TEM-1, TEM-2, SHV-1, and OXA-1, which results in the inhibition of bacterial cell wall synthesis and subsequent cell death. It also acts against non-ESBL class D oxacillinases. However, like other cephalosporins, it can be degraded by ESBLs and carbapenemases (29,61–64). Tazobactam protects ceftolozane from hydrolysis by irreversibly binding to most class A beta-lactamases (including the enzymes CTX-M, SHV and TEM) and some class C. As a result, it improves ceftolozane spectrum of activity against ESBL producing Enterobacteriaceae and some anaerobes (62) and broadening its antimicrobial effect. C/T has demonstrated minimal cross-resistance with other antimicrobials (61).

Currently C/T is approved at a dose of 1.5 g every 8 h as a 1 h rate of infusion (ceftolozane 1 g and tazobactam 0.5 g) for complicated urinary tract infections (cUTI) and complicated intra-abdominal infections (cIAI) in combination with

metronidazole and 3 g every 8 h (ceftolozane 2 g and tazobactam 1 g) for hospital-acquired bacterial pneumonia including ventilator-associated bacterial pneumonia (HABP/VABP) caused by Gram-negative organisms (65). However, the frequency and severity of MDR and XDR *P. aeruginosa* strains has led physicians to off-label use of C/T and hence it is generally reserved for the use against MDR/XDR *P. aeruginosa* strains (4,62,66). Pharmacodynamically, C/T is a time-dependent antibiotic and so the parameter that fits the best for predicting bacteriological efficacy is the percentage of the dosing interval in which the plasma free drug concentration remains higher than the minimum inhibitory concentration (%T>MIC), which in the case of C/T is approximately 40%–50% of the time between dosage administrations, similar to other cephalosporins (61,67).

Although CZA and C/T are generally reserved for MDR/XDR *P. aeruginosa* infections, the current standard dosing regimens could be insufficient when referring to MDR/XDR *P. aeruginosa* with MIC values close to susceptibility breakpoints, due to the increased likelihood of not achieving effective concentrations (68). In these scenarios, treatment with combination therapy or alternative dosing regimens need to be optimized and individualized (66) taking into account the patient's profile.

2.4. *In vitro* models

Combination antibiotic therapy has generated great interest in recent years because of the potential severity of infections due to XDR *P. aeruginosa* and the very high risk of selection of resistance. Various studies have examined *in vitro* interactions between bacteria and different antipseudomonal antibiotics, such as carbapenems, colistin and polymyxin B, fosfomycin, aminoglycosides and quinolones, using different methods to determine minimum inhibitory concentration (MIC) (for example, synergy testing using the microdilution checkerboard technique, gradient diffusion (Etest®) or time-kill curve assays.

These *in vitro* experiments could be static studies, such as checkerboard, or dynamic studies. Static systems can be used for quick determination of time killing

behaviour (53) meanwhile dynamic models provide more information under changing drug concentrations, their killing effect, the suppression of resistant mutant, dose fractionation, and also, combination therapy (27,69). About the dynamic models, they could be performed in one-compartment model (like time-kill curves or one-compartment *in vitro* model such as Chemostat) or in two-compartment model, such as the hollow-fiber infection system.

Time-kill curves allow similar antibiotics concentrations evaluation of that to those used in clinical practice, testing antibiotics alone or in combination, and adding time as a dynamic parameter (27). Some PD time-kill parameters were defined for combination therapies. Based on the final count of colonies in the antibiotic combination compared with the count for more effective of two components, it was defined additivity and synergy as a 1 to 2 $\log_{10}$ colony forming units or CFU/ml and as a $\geq$2 $\log_{10}$ CFU/ml, respectively (70,71).

The hollow-fiber infection model is a preclinical innovative method that makes possible to conduct experiments mimicking human pharmacokinetics under biosafety conditions (72). It could be a complement to or substitutes for animal models of infection, overcoming the limitations of static models. It is based on the use of hollow-fiber bioreactors, which are modules containing small tubular filters of 20 microns of diameter (73). There are specific advantages of the hollow-fiber infection model compared with static models. It allows analysing combination therapies, dosage profiles can be controlled over time, mechanisms of resistance can be revealed, and data is more clinically relevant, all without the restrictions of animal models. Compared with the one-compartmental model, it allows the bacterial load to remain constant, biosafety conditions for biohazardous organisms, and absorption, elimination and rapid antibiotic half-lives could be modelled (74). These nonclinical infection models can predict clinical outcomes (75,76). The insights gained from nonclinical infection models strongly support the rational design of optimal antibacterial dosage regimens for evaluation in future clinical trials (77). In relation to this, currently new antibiotics are not marketed without first being studied through PK/PD studies, and data obtained from these models are indispensable for selecting the doses and regimens for patients,

establishing susceptibility breakpoints, and ultimately refining clinical dosage regimens (77). Furthermore, the recruitment of a sufficient number of patients for clinical trials could be challenging. Consequently, these nonclinical PK/PD studies are required to support and enhance the insights gained from human studies (77).

Nevertheless, given the very high risk of selection for and spread of resistant mutants to drugs, it is of the utmost importance to monitor possible selection for resistance during treatment and the associated risk factors. One preventive measure would be to administer the new antimicrobials in specific forms and doses or in a more personalized way (taking into account the bacteria, type of infection and characteristics of the patient). This would prevent the development of resistance. In this regard, the hollow-fiber PK/PD dynamic model will enable us to discover which doses, routes of administration and antibiotics dosage would be the most effective and less likely to select resistant mutants during treatment for infections due to XDR *P. aeruginosa* clones. Consequently, the new drugs could be active for longer periods, which will benefit the patients.

3. OBJECTIVES

- MAIN OBJETIVE

The main objective of the present thesis is to evaluate different therapeutic options against extensively drug-resistant (XDR) *Pseudomonas aeruginosa*, including high-risk clones, using different *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) models.

- SPECIFIC OBJECTIVES

  - To evaluate various antipseudomonal antibiotics alone and in combination for the three most prevalent XDR *P. aeruginosa* high-risk clones (ST175, ST111 and ST235). To validate the most effective combination via checkerboard and time-kill curves in a representative collection of XDR *P. aeruginosa* isolates.

  - To study the combination of ceftolozane-tazobactam and colistin against the collection of clinical XDR *P. aeruginosa* isolates by means of the time-kill curve method. To validate the combination against three XDR *P. aeruginosa* isolates with different susceptibility levels to C/T in an *in vitro* Chemostat PK/PD model.

  - To evaluate the effectiveness of CZA alone and in combination with other antibiotics against XDR *P. aeruginosa* isolates via time-kill analysis.

  - To compare the efficacy of intermittent (1-h), extended (4-h) and continuous C/T infusion against three XDR *P. aeruginosa* ST175 isolates with different susceptibilities to C/T (MIC values between 2 and 16 mg/L) in an *in vitro* hollow-fiber infection model.

  - To assess the effectiveness and the emergence of resistance of alternative steady-state concentrations of C/T in CI against three XDR *P. aeruginosa* ST175 isolates in a hollow-fiber infection model.

4. PUBLICATIONS

4.1. Colistin plus meropenem combination is synergistic *in vitro* against extensively drug-resistant *Pseudomonas aeruginosa*, including high-risk clones.

Journal of Global Antimicrobial Resistance 2019; 18: 37-44.
DOI: 10.1016/j.jgar.2019.04.012.

Journal of Global Antimicrobial Resistance 18 (2019) 37–44

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Colistin plus meropenem combination is synergistic in vitro against extensively drug-resistant *Pseudomonas aeruginosa*, including high-risk clones

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ARTICLE INFO

Article history:
Received 7 January 2019
Received in revised form 29 March 2019
Accepted 24 April 2019
Available online 30 May 2019

Keywords:
Colistin
Meropenem
Combination therapy
Pseudomonas aeruginosa
Synergy

ABSTRACT

Background: Extensively drug-resistant (XDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) and particularly *P. aeruginosa* high-risk clones, are of growing concern because treatment options are limited. For years, colistin monotherapy has been the only available treatment, but is well known that is not an optimal treatment. A combination of colistin with another antibiotic could be a possible therapeutic option.
Objectives: This study aimed to investigate effective antibiotic combinations against 20 XDR *P. aeruginosa* isolates obtained in a Spanish multicentre study (2015).
Methods: Forty-five checkerboards with six antipseudomonal antibiotics (amikacin, aztreonam, ceftazidime, meropenem, colistin, and ceftolozane/tazobactam) were performed to determine whether combinations were synergic or additive by fractional inhibitory concentration indices. On average, 15 different regimens were evaluated in duplicate against the three most prevalent high-risk clones (ST175, ST235, ST111) by time-kill analyses over 24 h. The combination showing synergism in the three high-risk clones was validated in all studied XDR isolates.
Results: In time-kill curves, the untreated control failed, as did each study regimen when administered alone. Two combinations were synergistic in the three high-risk clones that were initially studied: amikacin plus ceftazidime and colistin plus meropenem, with the second being the most effective combination. The efficacy of colistin plus meropenem was then tested in all 20 isolates. A synergistic bacterial density reduction for the duration of the study occurred in 80% of the entire XDR collection.
Conclusions: These data suggest that colistin plus meropenem may be a useful combination for the treatment of infections due to XDR *P. aeruginosa*, including high-risk clones, which warrants evaluation in a clinical trial.
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1. Introduction

The world has faced a dramatic increase in antimicrobial resistance of Gram-negative bacteria in recent years. One representative microorganism with an extraordinary capacity to develop resistance is *Pseudomonas aeruginosa* (*P. aeruginosa*) [1,2]. Extensively drug-resistant (XDR) *P. aeruginosa* isolates, and

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https://doi.org/10.1016/j.jgar.2019.04.012
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particularly those recently designated as 'high-risk clones', are disseminated in hospitals around the world and have been related to very-difficult-to-treat infections [3–5]. The currently available therapeutic options for these infections yield suboptimal results, with concerning toxicity rates and a very narrow therapeutic window [6,7]. Without new therapeutic options, the outcome of patients with many types of infectious diseases will be compromised. Another worrisome feature of *P. aeruginosa* infection is the high risk for selection of resistant isolates during monotherapy [7]. These factors argue in favour of using combined therapy [5], which could broaden the spectrum of coverage, achieve an additive or synergistic antibacterial effect, and suppress emerging resistance [7].

Pseudomonas aeruginosa has a non-clonal epidemic population structure, in which a small number of widespread clones are selected from a background of a large number of rare and unrelated genotypes that recombine at a high rate [1]. In addition to classical molecular epidemiology and phenotypically-targeted assessment of resistance mechanisms, recent whole genome sequencing studies have provided relevant information regarding the complex resistome of multidrug-resistant (MDR)/XDR high-risk clones [8–14]. The most prevalent *P. aeruginosa* high-risk clones are thought to be ST111, ST175 and ST235 [1]. In a recent report, the current group analysed 150 XDR *P. aeruginosa* isolates from nine Spanish hospitals. Most of the isolates belonged to ST175 (67.3%), although ST244 (10.7%), ST235 (5.3%), and ST111 (1.3%) were also found. The remaining clones were less common and also less widely disseminated; these included ST253, ST313, ST179, ST274, ST395, ST455, ST2221, and four recently described STs: ST2533, ST2534, ST2535, and ST2536 [15].

Patients with infection caused by XDR *P. aeruginosa* high-risk clones are mainly treated with polymyxins or aminoglycosides [16]. Colistin use is hampered by the associated side effects (particularly nephrotoxicity) and difficulty in establishing an optimal dose and reaching therapeutic levels [6,16,17]. Polymyxin monotherapy may result in treatment failure (as reliably effective plasma exposure is not always attained), mainly relates to colistimethate, and bacterial resistance may emerge [18]. One solution for the scarcity of therapeutic options is to actively search for new strategies related to dosing, combining existing antibiotics, and developing new molecules. There is some hope in this line, as new molecules with antipseudomonal activity and new combinations with β-lactamase inhibitors are being developed. The clinical and microbiological impact of these new approaches against XDR *P. aeruginosa* high-risk clones is currently unknown; hence, clinical studies focusing on the treatment of these infections are urgently needed [19]. Furthermore, development of resistance to these new β-lactams has been recently reported [20].

Combination antibiotic therapy for XDR *P. aeruginosa* is generating interest because of the potential severity of the infection and the high risk of resistance selection with monotherapies. Several studies have examined in vitro interactions between various antipseudomonal antibiotics (e.g. carbapenems, colistin and polymyxin B, fosfomycin, aminoglycosides, and quinolones), using a variety of methods such as synergy testing using the microdilution checkerboard technique, gradient diffusion (Etest), and time-kill curve assays [7]. Nonetheless, no clear recommendations for clinical practice have emerged from these studies, and consensus is lacking as to which antibiotic combinations should be used against these complex infections to improve the therapeutic response and reduce selection of resistant mutants [21].

The primary aim of this study was to evaluate various antipseudomonal antibiotics alone and in combination for the three most prevalent XDR *P. aeruginosa* high-risk clones (ST175, ST111 and ST235) and to validate the most effective combination via checkerboard and time-kill curves in a collection of XDR isolates containing 20 representative isolates from a multicentre study.

2. Material and methods

2.1. Bacterial isolates

Twenty XDR *P. aeruginosa* clinical isolates were studied; they had been recovered in a recent study (COLIMERO study) in which 150 XDR *P. aeruginosa* isolates from nine Spanish hospitals were analysed using pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and whole-genome sequencing [15]. The 20 selected isolates were considered to provide a representative profile of all the clones and resistance mechanisms detected in the multicentre study.

2.2. Antibiotics

The antipseudomonal antibiotics used in the experiments were amikacin, aztreonam, ceftazidime, meropenem, colistin obtained from Sigma-Aldrich, and ceftolozane/tazobactam obtained from MSD (Merck Sharp & Dohme). Antibiotic solutions were prepared according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, using their corresponding solvent and dissolvent [22]. The doses stipulated for each antibiotic corresponded to the high doses used in clinical practice for the treatment of several infections. Antibiotic concentrations for time-kill experiments were chosen based on the area under the curve (AUC) serum levels: amikacin 1 g q24 h, AUC_{24}196 μg*h/mL [23,24]; aztreonam 2 g q8h, AUC_{24}1050 μg*h/mL [25]; ceftazidime 2 g q8h, AUC_{24} 800 μg*h/mL [26,27]; meropenem 2 g q8h, AUC_{24} 425 μg*h/mL [28]; colistin 4.5 MIU q12 h, AUC_{24} 50 μg*h/mL [29,30]; and ceftolozane/tazobactam 2/1 g q8h, AUC_{24} 912/150 μg*h/mL [31]. Colistin and meropenem concentrations in time-kill curves were validated by high performance liquid chromatography.

2.3. Susceptibility studies and resistance mechanisms

The susceptibility profiles and the β-lactam resistance mechanisms of the studied XDR isolates were obtained from a previous Spanish multicentre study [15]. The isolates accounted for the most prevalent and relevant resistance mechanisms, which included chromosomal mutations (AmpC hyperproduction and OprD inactivation) and horizontally acquired enzymes, including several metallo-β-lactamases (MBLs).

Antimicrobial susceptibility was performed according to the CLSI guidelines [22] for broth microdilution and agar dilution methods utilising cation-adjusted Mueller-Hinton broth (CAMHB).

2.4. Checkerboard experiments

Checkerboard studies were performed in 96-well microplates. The antibiotic values to be tested should include broad values ranging from 4–8 times the value of the expected MIC to at least 1/8 to 1/16 of it; 50 μL of CAMHB was distributed into each well. The first antibiotic solution was serially diluted and dispensed along the ordinate. The second antibiotic solution was diluted and dispensed along the abscissa. The bacteria inoculum equal to a 0.5 McFarland was prepared and 100 μL were distributed in each well. Plates were incubated at 35 °C for 48 h [32]. The fractional inhibitory concentration indices (FICIs) were calculated according to the following formula: FICI = $(IC_{A+B}/IC_A) + (IC_{A+B}/IC_B)$. The interaction was considered synergistic when FICI was $\leq$ 0.5,

additive when FICI was > 0.5 to ≤ 1, antagonistic when FICI was ≥ 4, and indifferent for intermediate values [33,34]. The experiments were performed in triplicate. Fifteen checkerboards were performed for each chosen strain. A representative strain of each of the three most prevalent high-risk clones — ST175, ST111 and ST235 — was evaluated.

2.5. Time-kill experiments

Time-kill studies were conducted with the six selected antibiotics alone and in combinations at clinically achievable drug concentrations (when maximum indicated clinical doses were used). Time-kill curves were performed with all the resulting combinations of the checkerboards, in each of the three isolates (ST175, ST111 and ST235). The most synergistic combination was then validated in the entire collection of XDR *P. aeruginosa* isolates, consisting of 20 isolates. All experiments were performed in duplicate. Study flow is represented in Fig. 1.

An overnight culture of isolate was diluted with CAMHB and further incubated at 35 °C to reach early log-phase growth. The bacterial suspension was diluted with CAMHB, according to its absorbance at 630 nm; 50 mL sterile conical flasks were used with 30 mL CAMHB. The final concentration of the bacterial suspension in each flask was approximately 7–8 $\log_{10}$ cfu/mL. Flasks were incubated in a shaker water bath at 35 °C for 24 h. Samples were collected from each flask at 0, 2, 4, 8, 12, and 24 h. The extracted broth samples (1 mL) were centrifuged twice at 5000 g for 5 min and then reconstituted with sterile saline solution to their original volumes to minimise drug carryover. Ten-fold serial dilutions were performed with CAMHB, 200 μL was plated on Muller Hinton E agar (MHE) plates, and total bacterial count was quantified for each sample. The inoculated plates were incubated in a humidified incubator (35 °C) for 18–24 h, bacterial colonies were visually counted, and the original bacterial density from the original sample was calculated based on the dilution factor. The limit of quantification (LOQ) was 400 CFU/mL (equivalent to 20 colonies per plate).

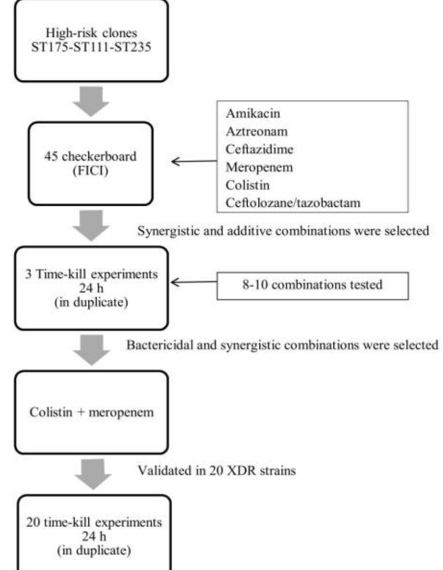

Fig. 1. Study flow.
Forty-five checkerboard screens with six selected antibiotics and with the three most prevalent *Pseudomonas aeruginosa* high-risk clones (ST175, ST111 and ST235) were conducted to identify additive and synergistic combinations. To ultimately identify 'the best combination', time-kill curves with the same clones were performed using an average of nine combinations previously detected on checkboard therapy. The combination selected was colistin plus meropenem, and this combination was validated in the entire collection (20 isolates) of extremely drug resistant bacteria.
FICI, fractional inhibitory concentration indice.

2.6. Pharmacodynamic checkpoints

Bactericidal activity was defined as a ≥ 3 $\log_{10}$ cfu/mL reduction in colony count at 24 h. Synergy was defined as a ≥ 2 $\log_{10}$ cfu/mL reduction in colony count at 24 h, with the combination as compared with the most active single drug. The combination was established as indifferent when there was a ≤ 2 $\log_{10}$ cfu/mL change at 24 h. Antagonism was defined as ≥ 1 $\log_{10}$ cfu/mL regrowth, with the combination as compared with the least active component [35].

3. Results

3.1. In vitro antimicrobial susceptibility testing and resistance mechanisms

The susceptibility profiles and resistance mechanisms are shown in the Table 1. The polymorphisms/mutations found in the genes related to colistin resistance in the 20 isolates of XDR *P. aeruginosa* are shown in the Table S1. All strains were resistant to meropenem, but three of them were intermediate (MIC 8 mg/L) according to CLSI definitions. ST111 (10-009) was resistant to colistin (MIC 4 mg/L), likely due to a 4-bp deletion within parR. All the other strains were susceptible (MIC ≤ 2 mg/L), and although amino acid polymorphisms within the main genes related with polymyxin resistance were detected in several strains [15], their effect, if any, would need to be specifically determined.

3.2. Checkerboard studies

In the checkerboard experiments, all selected combinations had a FICI within the range of 0.5–1; that is, only combinations that were synergistic (FICI ≤ 0.5) or additive (FICI > 0.5 ≤ 1). The combination colistin-meropenem was additive for the three most prevalent high-risk clones – ST175, ST111 and ST235 – in these experiments. These data are shown in the Table S2.

3.3. Time-kill studies

In the time-kill studies, growth in the untreated controls reached 9–10 $\log_{10}$ cfu/mL by the 24 h time point for all regimens. All isolates treated with single antibiotics (ceftazidime, aztreonam, meropenem, colistin, amikacin, or ceftolozane/tazobactam) did not show bactericidal effect after 24 h. The time-kill curves indicated that two combinations were synergistic in the three most prevalent high-risk isolates (ST175, ST111 and ST235): amikacin plus ceftazidime, and colistin plus meropenem, with the second being more effective (Table 2). When the colistin-meropenem combination was validated in time-kill studies including all 20 isolates, it was synergistic in 80% (Table 3, Fig. 2). Colistin-meropenem was not synergistic in four isolates, which surprisingly included three isolates with low MICs for meropenem (8 mg/L) — ST395/10-017, ST2534/06-025, and ST2535/06-027 — in which the monotherapy regimens showed results similar to those of the combination (Table 3, Fig. 2).

Table 1
Susceptibility profiles and resistance mechanisms of the 20 studied extremely drug resistant *Pseudomonas aeruginosa* isolates.

Isolate	ST	Beta -lactamases	AmpC hyper-production	OprD deficiency	Polymyxin resistance mechanisms	TOL/TZ	MER	CAZ	AZT	AMI	COL
04–025	175	–	Yes	Yes		2//4	16	32	16	4	1
10–009	111	VIM-2	Yes	Yes	*parR*- nt621Δ4	<64/4	<32	<64	<128	32	4
06–042	235	VIM-47	No	No		<64/4	<32	64	32	64	2
12–012	175	VIM-20, OXA-2	No	Yes		<64/4	<32	16	8	16	2
12–003	244	–	Yes	Yes		4/4	32	64	32	8	2
07–004	235	GES-19, OXA-2	No	Yes		<64/4	<32	<64	128	128	2
04–017	111	OXA-46	Yes	No		8/4	32	64	64	4	2
01–008	253	VIM-1	No	Yes		<64/4	<32	<64	4	8	2
10–023	175	–	Yes	Yes		2/4	16	32	16	4	2
07–016	175	GES-5	No	Yes		16/4	>32	32	16	16	2
09–007	313	–	Yes	Yes		4/4	16	64	32	8	2
06-035	455		Yes	No		4/4	>32	32	64	<2	0.5
06–014	179	OXA-10	Yes	Yes		4/4	32	16	16	8	2
10–019	2221		Yes	Yes		8/4	32	64	64	<2	2
10–021	2533		Yes	Yes		8/4	32	64	64	<2	1
06–001	2536		Yes	Yes		4/4	32	>64	64	8	2
09–011	274		Yes	Yes		8/4	32	64	64	128	1
06–025	2534		Yes	Yes		4/4	8	64	64	<2	2
06–027	2535		Yes	No		4/4	8	64	32	8	2
10–017	395		Yes	No		1/4	8	32	32	4	2

Minimal inhibitory concentrations (MICs) (mg/L) of the various antibiotics tested in this study: TOL/TZ, ceftolozane/tazobactam; MER, meropenem; CAZ, ceftazidime; AZT, aztreonam; AMI, amikacin; COL, colistin.

Table 2
Time-kill experiments performed against the three most prevalent extremely drug resistant *Pseudomonas aeruginosa* high-risk clones. A summary of mean bacterial concentrations (log_{10} CFU/mL) at 8, 12, and 24 h is shown for each strain and antibiotic treatment.

Isolate	ST	Inoculum	Antibiotic	8 h	12 h	SD12 h	24 h	SD24 h	Δ24 h	Synergy
04-025	**ST175**	7.19	Control	9.25	9.37	1.54	9.57	1.68	2.38	
			Amikacin	3.50	3.37	2.70	6.42	0.54	-0.77	
			Meropenem	3.21	3.83	2.37	5.18	1.42	-2.01	
			Ceftazidime	4.99	5.35	1.30	6.36	0.59	-0.83	
			Aztreonam	4.33	4.67	1.78	5.60	1.13	-1.59	
			Colistin	2.63	2.53	3.29	6.32	0.61	-0.87	
			Ceftolozane/tazobactam	4.65	4.79	1.70	5.28	1.35	-1.91	
			Amikacin + meropenem	2.90	2.97	2.99	4.03	2.23	-3.16	-1.15
			Amikacin + ceftazidime	2.76	2.65	3.21	3.37	2.70	-3.82	-3.05
			Amikacin + aztreonam	2.46	3.15	2.86	3.98	2.27	-3.21	-1.62
			Amikacin + ceftolozane/tazobactam	3.34	2.98	2.98	4.74	1.73	-2.45	-0.54
			Ceftolozane/tazobactam + meropenem	3.68	3.58	2.55	4.21	2.11	-2.98	-0.97
			Ceftolozane/tazobactam + ceftazidime	4.57	4.74	1.74	5.49	1.20	-1.70	0.21
			Ceftolozane/tazobactam + aztreonam	4.13	4.41	1.96	5.38	1.28	-1.81	0.10
			Ceftazidime + colistin	0.00	0.00	5.08	2.75	3.14	-4.44	-3.58
			Ceftazidime + aztreonam	4.46	4.92	1.61	5.70	1.06	-1.49	0.10
			Colistin + meropenem	0.00	0.00	5.08	2.03	3.65	-5.16	-3.16
10-009	**ST111**	6.94	Control	9.74	9.89	2.08	10.02	2.18	3.08	
			Amikacin	7.65	9.47	1.79	9.98	2.15	3.04	
			Meropenem	9.69	10.20	2.31	10.01	2.17	3.07	
			Ceftazidime	6.80	7.18	0.17	9.81	2.03	2.87	
			Aztreonam	6.97	7.20	0.18	7.41	0.33	0.47	
			Colistin	3.42	3.34	2.54	5.81	0.80	-1.13	
			Ceftolozane/tazobactam	9.60	10.03	2.18	10.00	2.16	3.06	
			Amikacin + meropenem	7.07	8.36	1.01	10.14	2.26	3.20	0.16
			Amikacin + ceftazidime	4.36	4.30	1.87	6.09	0.60	-0.85	-3.72
			Amikacin + aztreonam	3.69	4.44	1.76	4.15	1.97	-2.79	-3.26
			Amikacin + ceftolozane/tazobactam	5.47	6.40	0.38	10.22	2.32	3.28	0.24
			Ceftolozane/tazobactam + meropenem	8.91	9.52	1.82	9.93	2.11	2.99	-0.07
			Ceftolozane/tazobactam + ceftazidime	6.03	6.57	0.26	9.82	2.04	2.88	0.01
			Ceftazidime + colistin	1.34	2.16	3.38	2.49	3.15	-4.45	-3.32
			Colistin + meropenem	1.02	2.00	3.49	2.10	3.42	-4.84	-3.71
			Aztreonam + colistin	1.65	3.05	2.75	5.14	1.27	-1.80	-0.67
06-042	**ST235**	7.07	Control	9.70	8.84	1.25	9.88	1.98	2.81	
			Amikacin	9.54	9.91	2.01	9.97	2.05	2.90	
			Meropenem	4.07	4.84	1.58	9.98	2.06	2.91	
			Ceftazidime	7.50	7.83	0.54	10.03	2.09	2.96	
			Aztreonam	5.31	5.62	1.02	6.21	0.61	-0.86	
			Colistin	2.56	2.95	2.92	4.27	1.98	-2.80	
			Ceftolozane/tazobactam	9.43	9.58	1.77	10.11	2.15	3.04	
			Amikacin + meropenem	4.24	4.28	1.97	5.82	0.88	-1.25	-4.15
			Amikacin + ceftazidime	5.00	5.32	1.24	6.14	0.66	-0.93	-3.83
			Amikacin + aztreonam	4.75	5.12	1.38	5.34	1.23	-1.73	-0.87
			Amikacin + ceftolozane/tazobactam	7.20	7.30	0.16	9.90	2.00	2.83	-0.07
			Ceftolozane/tazobactam + meropenem	3.55	4.32	1.95	8.87	1.27	1.80	-1.11
			Ceftolozane/tazobactam + ceftazidime	6.57	7.82	0.53	10.09	2.14	3.02	0.07
			Ceftazidime + colistin	4.32	5.03	2.05	5.20	1.20	-1.70	0.93
			Colistin + meropenem	2.13	2.13	3.49	2.14	3.48	-4.93	-2.13

The standard deviation (SD) at 12 and 24 h, and change (**Δ**) in bacterial concentration in log_{10} CFU/mL at 24 h compared with the starting inoculum are shown. Bactericidal effect and synergy (≥3 log_{10} reduction in CFU/mL after 24 h and ≥ 2 log_{10} reduction in CFU/mL at 24 h with the combination as compared with the most active single drug, respectively) are highlighted in orange and yellow, respectively.

Table 3
Time-kill experiments performed against 20 extremely drug resistant *Pseudomonas aeruginosa* strains. Summary of mean bacterial concentration (log_{10}CFU/mL) at 8, 12 and 24 h is shown for each strain and antibiotic treatment.

Isolate	ST	Inoculum	Antibiotic	8 h	12 h	SD12 h	24 h	SD24 h	Δ24 h	Synergy
04-025	**175**	7.19	Control	9.25	9.37	1.54	9.57	1.68	2.38	
			Colistin	2.63	2.53	3.30	6.31	0.62	-0.88	
			Meropenem	3.21	3.83	2.38	5.18	1.42	-2.01	
			Colistin + meropenem	0	0	5.08	2.03	3.65	-5.16	-4.28
10-009	**111**	6.94	Control	9.74	9.89	2.08	10.02	2.18	3.08	
			Colistin	3.42	3.34	2.55	5.81	0.80	-1.13	
			Meropenem	9.69	10.2	2.31	10.01	2.17	3.07	
			Colistin + meropenem	1.02	2	3.49	2.1	3.42	-4.84	-3.71
06-042	**235**	7.07	Control	9.70	8.84	1.25	9.88	1.98	2.81	
			Colistin	2.56	2.95	2.91	4.27	1.98	-2.80	
			Meropenem	4.07	4.84	1.58	9.98	2.06	2.91	
			Colistin + meropenem	2.13	2.13	3.49	2.14	3.49	-4.93	-2.13
12-012	**175**	6.94	Control	9.82	10.14	2.26	9.81	2.02	2.87	
			Colistin	2.48	3.63	2.34	6.5	0.31	-0.44	
			Meropenem	9.44	9.76	1.99	9.88	2.08	2.94	
			Colistin + meropenem	1.75	1.69	3.71	3.6	2.36	-3.34	-2.9
12-003	**244**	6.99	Control	10.03	10.24	2.29	9.89	2.05	2.9	
			Colistin	5.28	6.4	0.42	6.9	0.06	-0.09	
			Meropenem	9.42	7.82	0.59	9.66	1.89	2.67	
			Colistin + meropenem	2.35	3.47	2.49	2.55	3.14	-4.44	-4.35
07-004	**235**	6.49	Control	9.69	10.04	2.51	10.05	2.52	3.56	
			Colistin	2.97	3.08	2.41	5.6	0.63	-0.89	
			Meropenem	9.61	10.02	2.50	9.86	2.38	3.37	
			Colistin + meropenem	2.12	2.87	2.56	3.26	2.28	-3.23	-2.34
04-017	**111**	7.03	Control	10.24	9.77	1.94	9.76	1.93	2.73	
			Colistin	2.32	3.22	2.69	5.51	1.07	-1.52	
			Meropenem	5.86	5.76	0.90	9.56	1.79	2.53	
			Colistin + meropenem	1.42	2.73	3.04	2.53	3.18	-4.5	-2.98
01-008	**253**	6.93	Control	9.79	9.82	2.04	10.15	2.27	3.22	
			Colistin	2.9	3.52	2.41	5.95	0.69	-0.98	
			Meropenem	9.78	9.86	2.07	10.15	2.28	3.22	
			Colistin + meropenem	2.21	1.95	3.52	3.8	2.21	-3.13	-2.15
10-023	**175**	7.0	Control	9.87	10.12	2.20	9.78	1.96	2.78	
			Colistin	3.69	4.06	2.08	8.09	0.77	1.09	
			Meropenem	4.5	4.54	1.74	7.93	0.66	0.93	
			Colistin + meropenem	2.84	3.07	2.78	4.35	1.87	-2.65	-3.58
07-016	**175**	6.92	Control	9.76	9.32	1.69	9.32	1.69	2.4	
			Colistin	3.25	4.56	1.67	6.55	0.26	-0.37	
			Meropenem	3.44	4.39	1.79	5.26	6.40	-1.66	
			Colistin + meropenem	2.51	2.09	3.42	2.88	2.86	-4.04	-3.67
09-007	**313**	6.9	Control	10.03	10.10	2.26	9.99	2.18	3.09	
			Colistin	3.56	3.31	2.54	7.68	0.55	0.78	
			Meropenem	3.85	3.79	2.20	5.39	1.07	-1.51	
			Colistin + meropenem	3.3	2.59	3.05	3.11	2.68	-3.79	-2.28
06-035	**455**	7.04	Control	9.96	10.02	2.11	9.86	1.99	2.82	
			Colistin	2.64	3.42	2.56	8.64	1.13	1.6	
			Meropenem	3.9	3.8	2.29	6	0.74	-1.04	
			Colistin + meropenem	2.28	3.24	2.69	2.96	2.88	-4.08	-3.04
06-014	**179**	7.02	Control	9.71	8.68	1.17	10.13	2.19	3.11	
			Colistin	1.85	2.83	2.96	4.82	1.56	-2.20	
			Meropenem	5.86	7.33	0.22	10.27	2.30	3.25	
			Colistin + meropenem	2.02	2.67	3.08	2.77	3.01	-4.25	-2.05
10-019	**2221**	7.16	Control	8.96	10.01	2.01	9.82	1.88	2.66	
			Colistin	2.44	4.21	2.09	6.35	0.57	-0.81	
			Meropenem	5.51	5.67	1.05	9.91	1.94	2.75	
			Colistin + meropenem	2.29	3.62	2.50	3.3	2.73	-3.86	-3.05
10-021	**2533**	7.22	Control	9.92	10.15	2.07	9.96	1.93	2.74	
			Colistin	3.48	3.23	2.82	5.13	1.48	-2.09	
			Meropenem	7.45	8.96	1.23	9.43	1.56	2.21	
			Colistin + meropenem	3.06	3.64	2.53	3.13	2.89	-4.09	-2
06-001	**2536**	6.8	Control	9.70	9.68	2.03	9.97	2.24	3.17	
			Colistin	3.94	4.02	1.97	6.89	0.06	0.09	
			Meropenem	5.77	6.75	0.04	9.56	1.95	2.76	
			Colistin + meropenem	3.14	3.58	2.28	3.28	2.49	-3.54	-3.61
09-011	**274**	7.13	Control	8.83	9.98	2.01	10.03	2.05	2.9	
			Colistin	1.71	3.05	2.88	3.8	2.35	-3.33	
			Meropenem	5.57	6.37	0.54	9.8	1.89	2.67	
			Colistin + meropenem	2.23	2.63	3.18	2.35	3.38	-4.78	-1.45
06-025	**2534**	7.07	Control	9.69	9.47	1.69	10.18	2.19	3.11	
			Colistin	3.66	4.29	1.97	5.54	1.08	-1.53	
			Meropenem	4.15	4.4	1.89	4.05	2.14	-3.02	
			Colistin + meropenem	3.77	4.27	1.98	2.78	3.03	-4.29	-1.27
06-027	**2535**	6.74	Control	10.08	9.61	2.02	9.82	2.17	3.08	
			Colistin	3.92	4.37	1.68	4.17	1.82	-2.57	
			Meropenem	3.94	4.72	1.43	2.8	2.79	-3.94	
			Colistin + meropenem	3.66	4.19	1.80	3.05	2.61	-3.69	0.25
10-017	**395**	6.92	Control	9.93	10.02	2.19	10.08	2.23	3.16	
			Colistin	3.15	3.62	2.33	4.5	1.71	-2.42	
			Meropenem	3.95	5.04	1.33	9.81	2.04	2.89	
			Colistin + meropenem	1.93	2.97	2.79	3.32	2.55	-3.6	-1.18

The standard deviation (SD) at 12 and 24 h, and the change (**Δ**) in bacterial concentration in log_{10}CFU/mL at 24 h compared with the starting inoculum are shown. Bactericidal effect and synergy ($\geq 3\ log_{10}$ reduction in CFU/mL after 24 h and $\geq 2\ log_{10}$ reduction in CFU/mL at 24 h with the combination as compared with the most active single drug, respectively) are highlighted in orange and yellow, respectively.

4. Discussion

This study evaluated the activity of antipseudomonal antibiotics used in clinical practice, alone and in combination, against representative XDR *P. aeruginosa* high-risk clones, with the aim of identifying the most effective antimicrobial combinations. The results open a new perspective in this line compared with the findings in previous studies. The combination with the greatest efficacy — colistin plus meropenem — showed a synergistic effect against 80% of the 20 strains that were studied, including those producing MBLs, one of which additionally showed colistin resistance and, therefore, panresistance.

Colistin-meropenem was not synergistic in four isolates, which surprisingly included three isolates with low MICs for meropenem. In two of three of the MIC of 8 to meropenem isolates the lacks of synergy appears to be due to the inability to improve markedly over meropenem monotherapy or are the results only valid when using drugs with beta lactams with compromised PK / PD that would be considered resistant. For the fourth isolate, the combination was not shown to be synergistic because of less than the required level of activity compared with colistin alone.

In previous studies, polymyxin-carbapenem combinations have been proposed for use in MDR Gram-negative infections to enhance the therapeutic response and minimise potential polymyxin resistance [7]. In the case of MDR *P. aeruginosa*, previous in vitro studies have found that colistin plus doripenem combination therapy is synergistic [7,8,18]. Other combinations with reported synergy against MDR *P. aeruginosa* include colistin-ceftazidime [36], colistin-rifampin [37,38], meropenem-levofloxacin [39], and colistin-imipenem [40]. The efficacy of the colistin-meropenem combination against XDR *P. aeruginosa* high-risk clones in the current study indicates that it may be a good option for infections, due to these difficult-to-treat bacteria. Although another synergistic combination – ceftazidime-amikacin – was found; it was decided to use the colistin-meropenem combination to test all 20 study isolates because the synergy values were better, and because the synergy between betalactamics and aminoglycosides has previously been studied more. As these infections often occur in patients with multiple conditions, including a risk of renal failure, the colistin-meropenem combination would be more useful in clinical practice because of its theoretically higher efficacy and lower risk of nephrotoxicity.

The mechanism of action of this combination is thought to be based on the combined effect of the two molecules on bacterial cells. Colistin acts against the lipopolysaccharide of the outer bacterial membrane, causing local disturbance, permeability changes, osmotic imbalance, and, usually, cell death [41]. Meropenem has to enter into the periplasmic space to the acetylate penicillin-binding proteins (PBPs), and interferes with the formation of peptidoglycan in the cell wall [42]. Mechanistically, colistin interferes with the outer membrane, changing its permeability, which in turn allows meropenem to enter the bacteria in higher amounts. Higher concentrations of meropenem in the periplasmic space could reduce the effect of resistance mechanisms, thereby rendering meropenem active against resistant bacteria. The classical mechanism of action of this combination (based on the permeability effect of colistin) has recently been complemented by new data from metabolomic studies in multidrug-resistant *Acinetobacter baumannii* treated with colistin

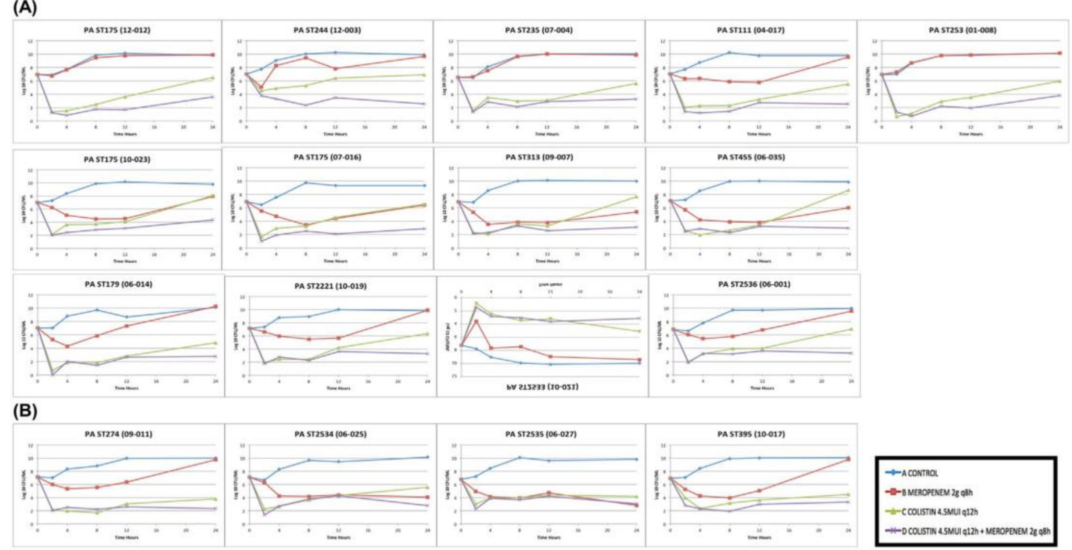

Fig. 2. Time-kill experiments displaying the activity of colistin and meropenem alone and in combination against the remainder (17/20) extremely drug resistant *Pseudomonas aeruginosa* strains. (A) Time-kill curve experiments for the 80% of the strains in which the combination showed a synergistic effect. (B) Time-kill curve experiments for the strains in which there was no synergistic effect.

plus doripenem [43]. Polymyxins and doripenem both interfere with key bacterial metabolic pathways in a time-dependent manner. In the reported experiments, colistin led to prompt inhibition of metabolic pathways (15 min–1 h), which was followed by the metabolic effects of doripenem at 4 h. This could explain the synergistic effect. Specifically, significant metabolic changes via disorganisation of membrane lipids and depletion of nucleotides, energy, and amino sugar metabolites were evident following treatment with colistin alone, and clearly enhanced by combining this drug with doripenem [43].

The phenomenon of bacterial regrowth shown in these single-drug experiments could be either due to a loss of functionality of these antibiotics or selection of resistant isolates. Presumably, the latter could include selection of pre-existing resistant subpopulations, de novo mutations, adaptive resistance, or formation of persistent cells [44]. Further studies would be required in order to evaluate these possibilities. It should be noted that bacterial regrowth is much more common in *P. aeruginosa* than in other Gram-negative bacteria when antimicrobial monotherapy is used. In this sense, combination therapy would not only enhance the antimicrobial effect, but also prevent the selection of resistant isolates [8], as was shown in the current study with the colistin-meropenem combination. Studies investigating resistance development with colistin monotherapy compared with combination therapy have shown suppression or delay of colistin resistance when combination therapy is used [7]. This is a part of the apparent success of combination therapy, and should be considered another argument in favour of using combinations in multidrug-resistant *P. aeruginosa* infection.

There are no clinical studies investigating colistin-meropenem in XDR *P. aeruginosa*. In a recently published clinical trial, the performance of the colistin-meropenem combination did not differ from that of colistin monotherapy against carbapenem-resistant Gram-negative bacteria. However, most of the infections that were included were due to *Acinetobacter baumannii* and no conclusions were obtained for MDR *P. aeruginosa* infections. [45]]. The current results indicate that clinical studies with MDR *P. aeruginosa* infection could be warranted to evaluate the colistin-meropenem combination.

This study had several limitations. Checkerboard studies were used only as screening, since it is a model with a fixed time and concentration and with low reproducibility [33,46]. Results provided by time-kill assays are more precise and sensitive for identifying possible synergies with combination regimens than checkerboard studies. Nevertheless, due to the differences in methodology and specific factors, it is hard to compare the different methods. Apart from that, antibiotic combinations were studied using fixed concentrations in time-kill studies. Since the interaction between antibiotics is dynamic and concentration-dependent [42], the results could vary if other concentrations were analysed. Furthermore, considering the usual posology in clinical practice, samples were obtained at different time points up to 12 h. Additionally, the curves were lengthened to 24 h to verify bacterial eradication, although the data obtained at that point cannot be considered relevant since they are not representative of the clinical administration guidelines for most antibiotics [33].

In summary, this study shows that the colistin-meropenem combination is bactericidal and synergistic against representative isolates of XDR *P. aeruginosa*. These results suggest that this therapy could be a potential option in severe infections caused by high-risk clones such as ST175, ST111, and ST235, including carbapenemase-producing and even panresistant isolates. Thus, this combination should be considered in future in vitro dynamic bi-compartmental studies and in clinical practice.

Funding

This study was partially supported by the Ministerio de Economía y Competitividad of Spain, Instituto de Salud Carlos III. FEDER (PI16/00669).

Competing interests

The authors declare no conflicts of interest.

Ethical approval

Not required.

Acknowledgements

We thank The Institute for Clinical Pharmacodynamics (ICPD), Schenectady, NY, and the Infectious Pathology and Antimicrobials Research Group (IPAR), Institute Hospital del Mar d' Investigacions Mèdiques (IMIM), for their support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.04.012.

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4.2. Efficacy of ceftolozane-tazobactam in combination with colistin against extensively drug-resistant *Pseudomonas aeruginosa*, including high-risk clones, in an *in vitro* pharmacodynamic model.

Antimicrobial Agents and Chemotherapy 2020; 64(4): e02542-19.
DOI: 10.1128/AAC.02542-19

EXPERIMENTAL THERAPEUTICS

Efficacy of Ceftolozane-Tazobactam in Combination with Colistin against Extensively Drug-Resistant *Pseudomonas aeruginosa*, Including High-Risk Clones, in an *In Vitro* Pharmacodynamic Model

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ABSTRACT Combination therapy is an attractive therapeutic option for extensively drug-resistant (XDR) *Pseudomonas aeruginosa* infections. Colistin has been the only treatment available for these infections for many years, but its results are suboptimal. Ceftolozane-tazobactam (C/T) is a newly available therapeutic option that has shown good antipseudomonal activity, even against a number of XDR *P. aeruginosa* strains. However, data about combinations containing C/T are scarce. The aim of this study was to analyze the activity of C/T and colistin alone and in combination against a collection of XDR *P. aeruginosa* strains containing 24 representative clinical isolates from a multicentre Spanish study. Twenty-four time-kill experiments performed over 24 h were conducted in duplicate to determine the effects of colistin and C/T alone and combined. An *in vitro* pharmacodynamic chemostat model then was used to validate this combination against three selected XDR *P. aeruginosa* ST175 isolates with different susceptibility levels to C/T. Static time-kill assays demonstrated superior synergistic or additive effect for C/T plus colistin against 21 of the 24 isolates studied. In the *in vitro* dynamic pharmacokinetic/pharmacodynamic (PK/PD) model, the C/T regimen of 2/1 g every 8 h with a steady-state concentration of 2 mg/liter colistin effectively suppressed the bacterial growth at 24 h. Additive or synergistic interactions were observed for C/T plus colistin against XDR *P. aeruginosa* strains and particularly against C/T-resistant strains. C/T plus colistin may be a useful treatment for XDR *P. aeruginosa* infections, including those caused by high risk-clones resistant to C/T.

KEYWORDS ceftolozane-tazobactam, colistin, combination therapy, *Pseudomonas aeruginosa*

Antibiotic resistance has existed since ancient times (1), but it is now a serious global health threat, responsible for over 0.7 million deaths each year (2). Bacteria can easily acquire new antibiotic resistance through chromosomal mutations and horizon-

Citation Montero M, Domene Ochoa S, López-Causapé C, VanScoy B, Luque S, Sorlí L, Campillo N, Angulo-Brunet A, Padilla E, Prim N, Pomar V, Rivera A, Grau S, Ambrose PG, Oliver A, Horcajada JP. 2020. Efficacy of ceftolozane-tazobactam in combination with colistin against extensively drug-resistant *Pseudomonas aeruginosa*, including high-risk clones, in an *in vitro* pharmacodynamic model. Antimicrob Agents Chemother 64:e02542-19. https://doi.org/10.1128/AAC.02542-19.

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Received 19 December 2019
Returned for modification 17 January 2020
Accepted 30 January 2020
Accepted manuscript posted online 10 February 2020
Published 24 March 2020

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tal gene transfer (3). *Pseudomonas aeruginosa* has an outstanding capacity to develop antibiotic resistance and is a leading cause of morbidity and mortality worldwide. This microorganism has a nonclonal epidemic population structure (4), and its antibiotic resistance can be caused by several mechanisms.

The recently designated high-risk *P. aeruginosa* clones are widely distributed in hospitals around the world and have been directly linked to severe, complex, and difficult-to-treat infections. ST111, ST175, and ST235 appear to be the most prevalent of these clones (4). ST175 is particularly common in several European countries, including Spain and France (5). It has been associated with multidrug-resistant (MDR) isolates and is a recognized hospital contaminant.

Patients with extensively drug-resistant (XDR) *P. aeruginosa* infections are at an increased risk of receiving inadequate initial antimicrobial therapy because of the limited treatment options available. Colistin is, and for many years has been, the only option available (6). Its use, however, is limited by nephrotoxicity (7–10), difficulties achieving therapeutic levels, and heteroresistance (11). Data from pharmacokinetic studies have confirmed that colistin plasma concentrations following EMA and FDA dosage recommendations are low and inadequate for the treatment of MDR/XDR *P. aeruginosa* infections (12). Thus, there is clearly a need to investigate the performance of colistin combined with other antibiotics in *P. aeruginosa* infections (13).

Ceftolozane-tazobactam (C/T) has emerged as a promising solution to the lack of new antibiotics that are effective against *P. aeruginosa* (14). C/T is generally reserved for MDR/XDR *P. aeruginosa* infections, but there are clinical situations, including XDR *P. aeruginosa* infections with a C/T MIC of >4 mg/liter (C/T-nonsusceptible isolates), in which treatment needs to be optimized, probably through combination therapy (15).

Several studies have recommended antibiotic therapy combining an antipseudomonal β-lactam and an aminoglycoside or fluoroquinolone (16–20). Data on combinations containing C/T, however, are scarce (21–24). Combination regimens featuring colistin are more common and were recently recommended in the IDSA guidelines for the treatment of critically ill patients with ventilator-associated pneumonia due to the high prevalence of MDR/XDR microorganisms (25).

This study aimed to evaluate the antibacterial activity of C/T and colistin alone and in combination against a representative collection of clinical XDR *P. aeruginosa* strains in an *in vitro* pharmacodynamic model simulating the free drug concentration achieved with recommended dosing regimens for each antibiotic.

RESULTS

***In vitro* antimicrobial susceptibility studies.** All of the isolates were tested for antibiotic susceptibility. Twelve (ST111 [10-009], ST235 [06-042], ST175 [12-012], ST235 [07-004], ST111 [04-017], ST253 [01-008], ST175 [07-016], ST2221 [10-019], ST2533 [10-021], ST274 [09-011], ST175 [15-001], and ST175 [09-012]) were resistant to C/T. Seven of the 12 C/T-resistant isolates harbored carbapenemases. Only one isolate, ST111 (10-009), was resistant to colistin. The susceptibility profiles and the β-lactam resistance mechanisms of the XDR isolates studied were obtained from a previous Spanish multicenter study (26) and are shown in Table 1.

Three of the previous isolates were selected to perform a one-compartment *in vitro* chemostat model: ST175 (10-023), ST175 (09-012), and ST175 (07-016).

ST175 (10-023) was C/T susceptible, with a MIC of 2 mg/liter, and showed the typical resistance profile associated with this clone (resistance to all β-lactams except C/T) due to OprD inactivation and AmpC hyperproduction. ST175 (09-12) showed intermediate resistance to C/T with a MIC of 8 mg/liter. In addition to OprD inactivation and AmpC hyperproduction, it showed a specific mutation in PBP3 (R504C) associated with increased β-lactam resistance. Finally, ST175 (07-016) was C/T resistant with a MIC of 16 mg/liter and produced the class A carbapenemase GES-5.

Time-kill studies. In the time-kill assays, colistin and C/T alone were not bactericidal in 83% and 87.5% of the samples, respectively. However, the combination of C/T plus colistin was synergistic in 54% of isolates and additive in 87.5%. The combination

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TABLE 1 Susceptibility profiles and resistance mechanisms of the 24 XDR *P. aeruginosa* isolates studied

						MIC (mg/liter) for[a]:	
Isolate	ST	β-Lactamase(s)	AmpC hyperproduction	OprD deficiency	Polymyxin resistance mechanism	C/T	CST
04-017	111	OXA-46	Yes	No		8/4	2
10-009	111	VIM-2	Yes	Yes	*parR*-nt621Δ4	>64/4	4
04-025	175		Yes	Yes		2/4	1
07-016	175	GES-5	No	Yes		16/4	2
10-023	175		Yes	Yes		2/4	2
12-012	175	VIM-20, OXA-2	No	Yes		>64/4	2
06-014	179	OXA-10	Yes	Yes		4/4	2
07-004	235	GES-19, OXA-2	No	Yes		>64/4	2
06-042	235	VIM-47	No	No		>64/4	2
12-003	244		Yes	Yes		4/4	2
01-008	253	VIM-1	No	Yes		>64/4	2
09-011	274		Yes	Yes		8/4	1
09-007	313		Yes	Yes		4/4	2
10-017	395		Yes	No		1/4	2
06-035	455		Yes	No		4/4	0.5
10-019	2221		Yes	Yes		8/4	2
10-021	2533		Yes	Yes		8/4	1
06-025	2534		Yes	Yes		4/4	2
06-027	2535		Yes	No		4/4	2
06-001	2536		Yes	Yes		4/4	2
04-024	175		Yes	Yes		4/4	2
12-017	175		Yes	Yes		4/4	2
15-001	175		Yes	Yes		8/4	2
09-012	175		Yes	Yes		8/4	2

[a]Abbreviations: C/T, ceftolozane-tazobactam; CST, colistin.

regimen was efficacious for both colistin and C/T alone in 21 of the 24 isolates studied, and no antagonism was observed for any of the isolates. The results are shown in Table 2. The Kruskal-Wallis test showed statistically significant differences between the conditions studied ($X^2 = 57.52$, gl = 3, $P < 0.001$). Post hoc tests allow us to affirm that $\mu A > (\mu B = \mu C) > \mu D$; therefore, the *in vitro* combined treatment of C/T and colistin is more effective than monotherapy with any of them. In the time-kill studies, growth in the nontreatment controls reached 9 to 10 $\log_{10}$ CFU/ml by the 24-h time point for all regimens.

Time-kill curves with colistin alone showed a similar pattern in all the isolates, with an initial 3 to 5 $\log_{10}$ reduction after 2 h, followed by regrowth in all cases. In 11 isolates (ST111 [10-009], ST175 [10-023], ST175 [12-012], ST313 [09-007], ST395 [10-017], ST455 [06-035], ST2534 [06-025], ST2536 [06-001], ST175 [04-024], ST175 [12-017], and ST175 [15-001]), bacterial regrowth occurred, reaching concentrations similar to those of the controls. A bactericidal effect was observed for colistin in four isolates (ST235 [06-042], ST274 [09-011], ST2535 [06-027], and ST175 [09-012]). Three different behaviors were observed for C/T: five isolates (ST111 [10-009], ST175 [12-012], ST235 [07-004], STS35 [06-042], and ST235 [01-008]) showed the same behavior as the untreated control; 16 isolates showed a 2- to 4-$\log_{10}$ CFU/ml reduction at 8 h, followed by a plateau in 14 cases; and the remaining isolate, ST2534 (06-025), showed a 4-$\log_{10}$ CFU/ml increase at 24 h. A bactericidal effect was detected in five isolates (ST175 [07-023], ST2533 [10-021], ST2535 [06-027], ST2536 [06-001], and ST175 [09-012]).

All isolates treated with the C/T plus colistin combination showed an initial 2- to 5-$\log_{10}$ CFU/ml reduction followed by a plateau. The combination had bactericidal effect in all cases, a synergistic effect at 24 h in 13 isolates (ST111 [04-017], ST111 [10-009], ST175 [10-023], ST175 [12-012], ST179 [06-014], ST244 [12-003], ST313 [09-007], ST395 [10-017], ST2534 [06-025], ST2536 [06-001], ST175 [04-024], ST175 [12-017], and ST175 [15-001]), and an additive effect at 24 h in all but three isolates (ST 2533 [10-021], ST 2535 [06-027], and ST175 [09-012]). In this last case, the results were similar to those observed for C/T and colistin as monotherapy. No antagonism was detected.

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TABLE 2 Time-kill experiments performed against 24 XDR *P. aeruginosa* strains[a]

Isolate	Atb	0h	2h	4h	8h	12h	24h	Bact.E ΔCFU/mL	Syn./Addi. 24h ΔCFU/mL
ST111 (04-017)	CST	6.91	1.54	1.76	2.73	2.87	5.77	-1.14	
	C/T	6.91	6.06	6.04	4.04	4.26	4.81	-2.1	
	C/T+CST	6.91	1.68	1.36	3.36	2.79	2.08	-4.83	-2.73
ST111 (10-009)	CST	6.67	5.58	5.99	7.74	8.70	9.77	3.10	
	C/T	6.67	6.27	8.02	8.89	9.58	10.1	3.43	
	C/T+CST	6.67	4.91	3.55	4.47	4.47	3.67	-3.00	-6.1
ST175 (04-025)	CST	7.01	1.72	2.15	1.99	3.22	5.50	-1.51	
	C/T	7.01	6.00	4.65	3.77	4.34	4.09	-2.92	
	C/T+CST	7.01	2.10	1.73	2.29	3.38	3.05	-3.96	-1.04
ST175 (07-016)	CST	6.90	2.59	1.66	3.35	4.58	6.31	-0.59	
	C/T	6.90	5.26	5.00	4.04	4.76	5.04	-1.86	
	C/T+CST	6.90	2.35	2.97	4.00	3.86	3.65	-3.25	-1.39
ST175 (10-023)	CST	6.86	3.37	2.18	3.41	3.91	7.63	0.77	
	C/T	6.86	6.02	5.64	4.77	4.94	5.03	-1.83	
	C/T+CST	6.86	3.58	1.75	3.87	4.26	3.46	-3.40	-1.57
ST175 (12-012)	CST	6.87	2.31	2.52	3.98	4.11	9.39	2.52	
	C/T	6.87	6.63	7.43	8.87	9.40	9.18	2.31	
	C/T+CST	6.87	1.15	2.03	3.10	3.05	3.73	-3.14	-5.45
ST179 (06-014)	CST	7.18	0.94	2.24	2.41	3.17	5.41	-1.77	
	C/T	7.18	5.09	5.01	4.04	4.05	4.52	-2.66	
	C/T+CST	7.18	0.85	1.26	2.57	3.53	2.02	-5.16	-2.5
ST235 (07-004)	CST	7.07	1.95	1.74	1.88	2.77	5.54	-1.53	
	C/T	7.07	7.04	8.11	9.85	9.83	10.0	2.93	
	C/T+CST	7.07	1.54	1.57	2.97	2.62	4.07	-3.00	-1.47
ST235 (06-042)	CST	7.29	2.53	1.99	1.76	3.05	4.06	-3.23	
	C/T	7.29	5.92	7.03	9.01	9.67	10.0	2.71	
	C/T+CST	7.29	2.31	2.25	1.85	3.17	3.06	-4.23	-1.00
ST244 (12-003)	CST	6.98	1.54	1.97	5.08	5.56	7.65	0.67	
	C/T	6.98	6.23	4.65	5.28	5.69	5.04	-1.94	
	C/T+CST	6.98	1.24	1.51	3.92	3.88	2.98	-4.00	-2.06
ST253 (01-008)	CST	7.09	0.35	1.17	3.67	3.89	5.27	-1.82	
	C/T	7.09	6.66	8.50	9.70	9.85	10.2	3.11	
	C/T+CST	7.09	1.00	1.81	2.74	3.92	3.81	-3.28	-1.46
ST274 (09-011)	CST	7.16	1.59	2.10	2.38	3.28	3.60	-3.56	
	C/T	7.16	6.43	6.26	3.27	4.58	5.18	-1.98	
	C/T+CST	7.16	1.36	1.83	2.00	3.11	2.60	-4.56	-1.00
ST313 (09-007)	CST	6.86	3.22	1.87	3.63	4.17	8.59	1.73	
	C/T	6.86	6.13	5.62	4.28	4.59	4.65	-2.21	
	C/T+CST	6.86	2.83	2.36	3.00	3.80	3.63	-3.23	-1.02
ST395 (10-017)	CST	6.96	3.35	4.03	6.65	7.26	8.77	1.81	
	C/T	6.96	6.18	5.13	4.15	4.25	4.29	-2.67	
	C/T+CST	6.96	2.70	1.88	1.09	3.04	2.27	-4.69	-2.02
ST455 (06-035)	CST	6.97	3.74	3.39	3.51	3.69	8.97	2.00	
	C/T	6.97	6.03	5.85	4.45	4.60	4.32	-2.65	
	C/T+CST	6.97	2.78	2.03	2.23	2.46	2.03	-4.94	-2.29
ST2221 (10-019)	CST	6.91	1.68	1.66	2.76	2.98	5.07	-1.84	
	C/T	6.91	6.18	5.45	3.90	4.49	4.57	-2.34	
	C/T+CST	6.91	1.33	1.13	2.81	3.01	2.44	-4.47	-2.13
ST2533 (10-021)	CST	6.98	0.85	3.01	4.07	2.50	4.52	-2.46	
	C/T	6.98	6.51	5.71	3.54	3.90	3.84	-3.14	
	C/T+CST	6.98	0.50	1.45	1.39	2.98	2.92	-4.06	-0.92

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Antibiotic exposures. The observed concentrations and pharmacokinetic parameters calculated for all antibiotic regimens over the 24 h of the chemostat experiments are shown in Table 3. Overall, the observed versus predicted drug exposures of ceftolozane achieved in this model were considered satisfactory for all regimens based on observed r^2 values of 0.97, 0.96, and 0.94 for ST175 (10-023), ST175 (09-12), and ST175 (07-016), respectively (Fig. 1).

Chemostat studies. In the *in vitro* pharmacokinetics/pharmacodynamics model, the C/T regimen of 2/1 g every 8 h (q8h) combined with steady-state concentrations of 2 mg/liter colistin effectively suppressed the bacterial growth at 24 h. Additive and synergistic interactions were observed for C/T plus colistin against XDR *P. aeruginosa* strains and particularly against strains that were resistant to C/T. These results are shown in Fig. 2.

TABLE 2 (Continued)

Isolate	Atb	0h	2h	4h	8h	12h	24h	Bact.E ΔCFU/mL	Syn./Addi. 24h ΔCFU/mL
ST2534 (06-025)	CST	7.09	1.78	1.64	2.15	3.75	7.68	0.59	
	C/T	7.09	5.51	5.43	4.37	3.88	7.57	0.48	
	C/T+CST	7.09	1.51	1.44	2.00	3.37	2.42	-4.67	-5.15
ST2535 (06-027)	CST	6.97	0.50	1.13	1.03	3.07	2.88	-4.09	
	C/T	6.97	6.63	6.14	3.93	3.65	3.16	-3.81	
	C/T+CST	6.97	2.22	1.65	1.89	3.10	3.21	-3.76	0.33
ST2536 (06-001)	CST	7.13	2.23	1.99	2.54	4.50	8.61	1.48	
	C/T	7.13	5.12	4.79	3.40	3.67	4.17	-2.96	
	C/T+CST	7.13	1.66	1.14	3.07	2.18	1.63	-5.5	-2.54
ST175 (04-024)	CST	7.00	1.70	2.48	2.32	4.00	8.00	1.00	
	C/T	7.00	6.78	5.40	4.10	4.18	4.48	-2.52	
	C/T+CST	7.00	1.60	0.00	2.30	2.18	2.20	-4.8	-2.28
ST175 (12-017)	CST	7.23	1.93	1.00	2.16	3.40	7.69	0.46	
	C/T	7.23	7.06	6.15	6.15	4.70	5.04	-2.19	
	C/T+CST	7.23	2.15	2.15	0.70	2.67	2.43	-4.8	-2.61
ST175 (15-001)	CST	7.02	1.88	1.98	2.81	3.69	8.04	1.02	
	C/T	7.02	6.30	6.65	5.40	4.74	4.60	-2.42	
	C/T+CST	7.02	1.60	1.60	0.00	1.93	2.15	-4.87	-2.45
ST175 (09-012)	CST	6.81	1.40	1.88	1.18	2.49	3.34	-3.47	
	C/T	6.81	6.27	5.11	4.70	3.40	3.74	-3.07	
	C/T+CST	6.81	1.18	0.00	0.00	2.18	2.83	-3.98	-0.51

[a]Bactericidal effect (≥3-$\log_{10}$ reduction in CFU/ml after 24 h) is highlighted in blue. Synergistic and additive effects (≥2-$\log_{10}$ or ≥1-$\log_{10}$ reduction in CFU/ml at 24 h with the combination compared with the most active single drug) are highlighted in orange and green, respectively. Abbreviations: Atb, antibiotic; CST, colistin; C/T, ceftolozane-tazobactam. Mean bacterial concentration ($\log_{10}$ CFU/ml) is shown for each strain and antibiotic treatment. Positive results for bactericidal effect (Bact.E) and synergy at 24 h (Syn.24h) are highlighted.

In the C/T-susceptible ST175 (10-023) isolate, the addition of colistin to C/T resulted in a significant reduction in number of CFU compared with that of either antibiotic alone. There was a 1.74-$\log_{10}$ CFU/ml difference in the reduction achieved with the more active antibiotic (C/T); therefore, the combination was deemed to have an additive effect.

In the intermediate-resistant C/T ST175 (09-12) isolate, C/T monotherapy resulted in a 2.41-$\log_{10}$ CFU/ml reduction at 24 h. Nevertheless, the addition of colistin resulted in a significant reduction in bacterial burden compared with that for either antibiotic alone. Colistin monotherapy resulted in strong regrowth at 24 h. The difference of 2.57

TABLE 3 Observed concentrations and calculated pharmacokinetic parameters calculated for all antibiotic regimens over 24 h in the chemostat experiments[c]

	Free peak concn (mg/liter)		Free trough concn (mg/liter)	
Isolate and regimen	Target	Observed [means (SD)]	Target	Observed [means (SD)]
ST175 (10-023)				
C/T[a]	87.6	85.39 (14.71)	17.4	15 (2.42)
Colistin[b]	1	1.90 (1.15)	NC	NC
ST175 (09-012)				
C/T	87.6	112.8 (8.36)	17.4	30.6 (5.29)
Colistin	1	1.08 (0.33)	NC	NC
ST175 (07-016)				
C/T	87.6	121.7 (12.05)	17.4	25.5 ± 8.91
Colistin	1	1.72 (0.38)	NC	NC

[a]Concentration and pharmacokinetic data were targeted only for the ceftolozane component of C/T.
[b]Colistin was administered as a continuous infusion; the free peak concentration is the mean steady-state concentration over the 24 h of the experiment.
[c]Data are presented as the means and standard deviations. Abbreviations: C/T, ceftolozane-tazobactam; NC, not calculated.

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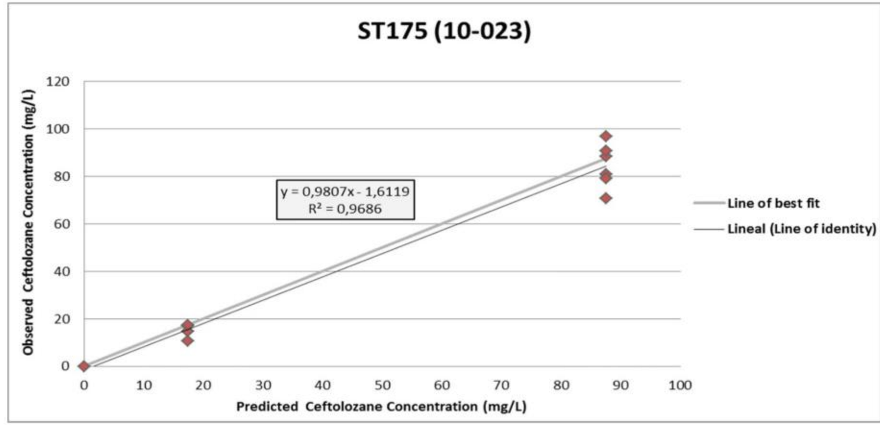

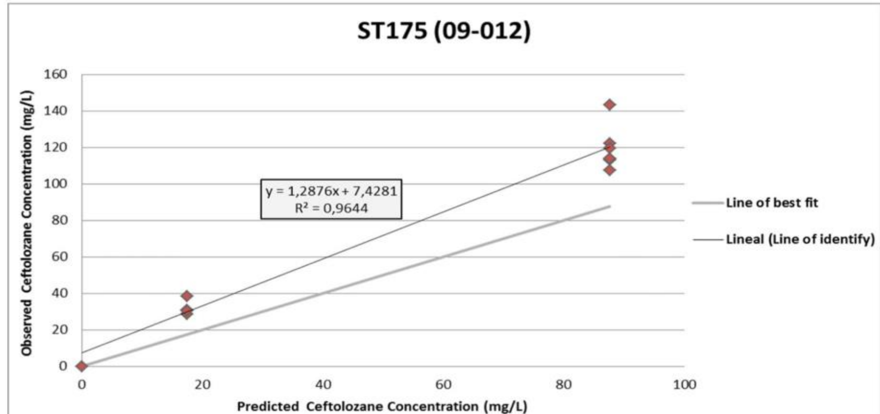

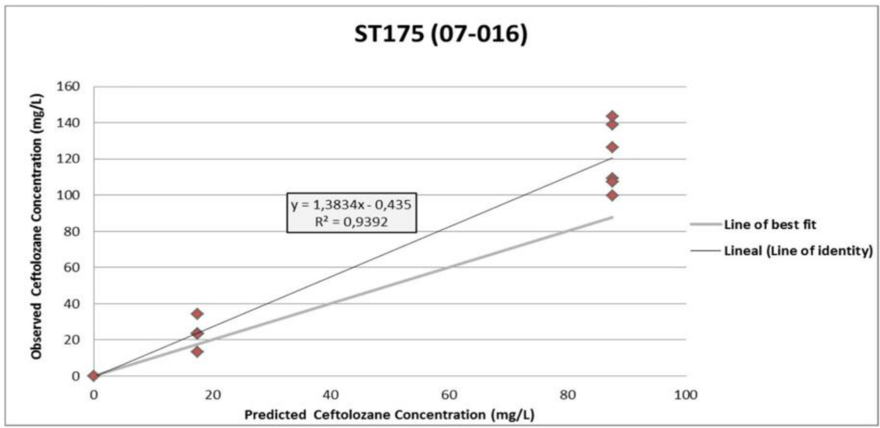

FIG 1 Relationships between observed and targeted ceftolozane concentrations for the three selected XDR *P. aeruginosa* ST175 isolates: ST175 (10-023), ST175 (09-012), and ST175 (07-016).

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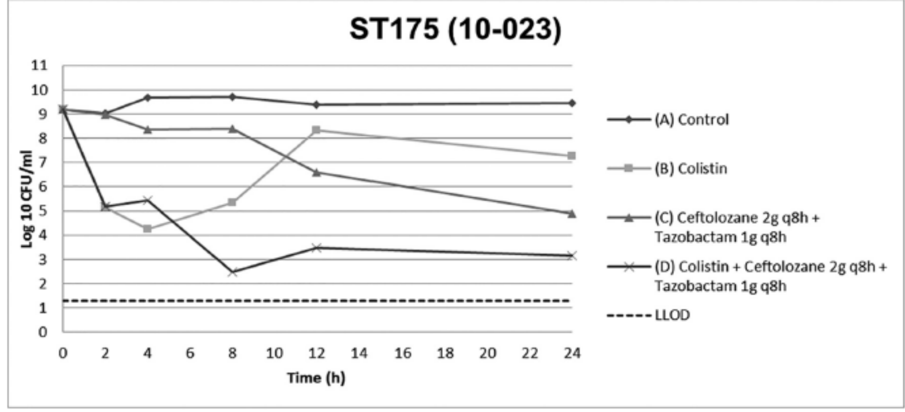

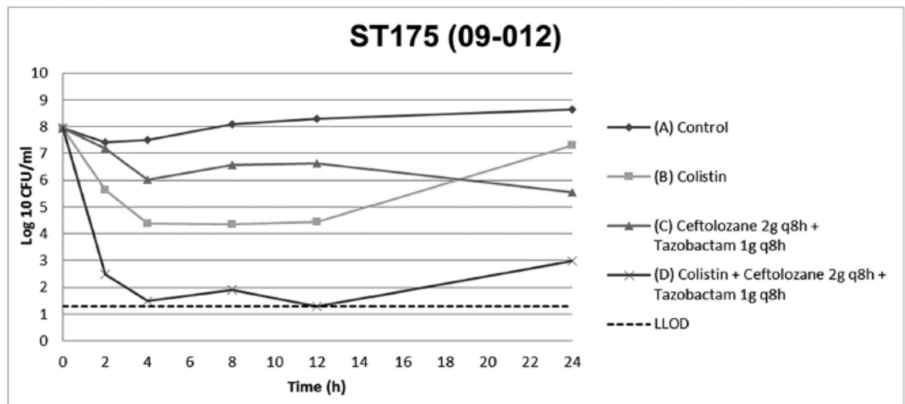

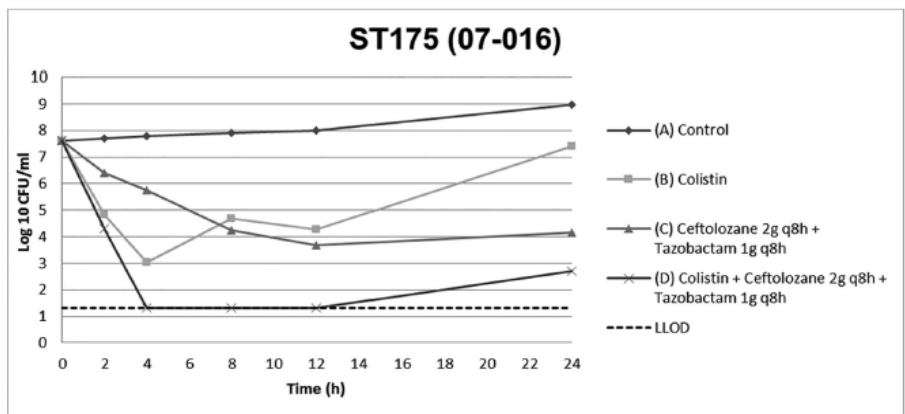

FIG 2 *In vitro* chemostat experiments with three selected XDR *P. aeruginosa* ST175 isolates with different susceptibility levels to C/T: ST175 (10-023), with a MIC of 2 mg/liter, ST175 (09-012), with a MIC of 8 mg/liter, and ST175 (07-016), with a MIC of 16 mg/liter. Values shown are mean numbers of CFU over 24 h for each *P. aeruginosa* isolate and antibiotic. LLOD, lower limit of detection.

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TABLE 4 *In vitro* chemostat results

	ST175 (10-023)		ST175 (09-012)		ST175 (07-016)	
	Log diff 24 h[a]	LR of AUCFU[b]	Log diff 24 h	LR of AUCFU	Log diff 24 h	LR of AUCFU
CST vs Control	-2.18	-1.34	-1.35	-1.38	-1.55	-1.55
C/T vs Control	-4.61	-1.16	-3.1	-1.45	-4.81	-2.17
C/T + CST vs CST alone	-4.12	-0.34	-4.32	-0.37	-4.7	-0.68
C/T + CST vs C/T alone	-1.74	-0.51	-2.57	-0.29	-1.44	-0.10

[a]Log difference at 24 h for each antibiotic alone compared with the control and for each antibiotic combination compared with each antibiotic alone. Synergy and additive effect (≥2-$\log_{10}$ or ≥1-$\log_{10}$ reduction in CFU/ml at 24 h with the combination compared with the most active single drug) are highlighted in orange and green, respectively.

[b]The log difference is presented as the log ratio (LR), which is used to compare any number of $\log_{10}$ CFU of two regimens (test/reference). AUCFU, area under the curve for CFU.

$\log_{10}$ CFU/ml achieved with the combination compared with the more active antibiotic (C/T) meant that the combination was classified as synergistic.

ST175 (07-016) was C/T resistant. Nevertheless, C/T monotherapy resulted in a 3.46-$\log_{10}$ CFU/ml reduction at 24 h, but its bactericidal effect was low compared with that of the combination regimen. C/T plus colistin resulted in a significant reduction of 1.44 $\log_{10}$ CFU/ml compared with the most active single drug (C/T), qualifying this combination as additive.

As an alternative endpoint, for each regimen (including the growth control) for the duration of the study, we calculated the log ratio (LR) for the area under the curve for CFU (AUCFU) as the total bacterial exposure. Relative to the control (reference), all monotherapies achieved greater than 1-log reduction against the three isolates, including a 2-log reduction achieved with C/T compared with the reference in the ST175 (07-016) isolate. AUCFU reductions with the combination regimens were <1 log relative to the reduction achieved with each antibiotic alone in the three isolates studied.

Table 4 shows *in vitro* chemostat results as log difference at 24 h and LR of AUCFU for each antibiotic alone (test) compared with the control (reference) and for each antibiotic combination (test) compared with each antibiotic alone (reference).

Resistance studies. In the chemostat model, C/T-resistant strains were not found with C/T alone or in combination with colistin over the 24 h of the experiments. The emergence of a colistin-resistant subpopulation was detected at the end of the experiment in the control and in the chemostat cultures receiving colistin alone. No colistin-resistant subpopulations were detected for the combination of C/T plus colistin. The frequency of the colistin-resistant subpopulation at concentrations of 2-fold the MIC was 1 CFU/ml in 3.26×10^{11}, 4.08×10^{8}, and 3.84×10^{7} CFU/ml for ST175 (10-023), ST175 (09-12), and ST175 (07-016), respectively. At concentrations 4-fold the MIC, the respective frequencies were 1 CFU/ml in 3.8×10^{11}, 1.37×10^{8}, and 2.67×10^{8} CFU/ml.

DISCUSSION

C/T emerged as a beacon of hope for the treatment for MDR/XDR Gram-negative bacteria, and it is one of the latest additions to the antibiotic armamentarium for treating severe infections caused by *P. aeruginosa*. In routine practice, however, there are many patients with particularly severe infections, or with a MIC for the MDR/XDR *P. aeruginosa* strains tested that is above the susceptibility breakpoint, who could benefit from combination therapy with C/T and colistin. Another reason for using this combination is to prevent the development of resistance, especially to β-lactams.

Our study evaluated combination therapy with C/T and colistin in a large collection of representative XDR *P. aeruginosa* isolates, including prevalent high-risk clones. The 24 isolates selected were resistant to all the β-lactams tested, and 12 of them (50%) were also resistant to C/T, with 7 of them harboring carbapenemases. Just one isolate (ST111) was resistant to colistin. We selected these highly resistant strains because they

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cause precisely the type of infections that could benefit from combination therapy. Few studies have examined combination therapy of C/T plus colistin. Monogue and Nicolau (27) published the first study assessing synergy of C/T against Gram-negative microorganisms and demonstrated synergistic effects for C/T combined with colistin or fosfomycin using time-kill curves. Rico Caballero et al. (24) further added to this body of knowledge in an *in vitro* pharmacodynamic study that showed greater overall reductions in bacterial burden and additive or synergistic effects for C/T combined with amikacin or colistin.

Time-kill curves were generated to evaluate the effects of C/T and colistin alone and in combination in all of the isolates. Colistin showed a similar pattern across the isolates (initial 3- to 5-log reduction in bacterial growth after 2 h, followed by regrowth in all cases), supporting previous reports (24).

The combination of colistin and C/T led to a rapid and sharp decrease in bacterial burden in all isolates but one, regardless of C/T resistance. A moderate decrease was also observed for C/T plus colistin for the isolate resistant to both antibiotics. Following the initial decrease in bacterial burden, there was a plateau in the curves with this antibiotic combination. In disagreement with other reports (21, 24, 27) and in contrast to when colistin was used as a monotherapy, no regrowth was observed for C/T plus colistin for any of the isolates. C/T combined with colistin had a bactericidal effect in all of the isolates. The combination was synergic at 24 h in 13 isolates and additive in an additional eight. Interestingly, synergy was observed in five of the non-C/T-resistant isolates, two of which had a MIC of ≥64 mg/liter. The aforementioned rapid killing caused by colistin probably contributed to the synergistic effect observed for this antibiotic combination, even in some of the C/T-resistant isolates. This effect is probably due to the different mechanisms of action of these two antibiotics (28), and their combined effect on bacterial cells would explain the mechanism of action of the combination. Colistin acts against the lipopolysaccharide in the outer membrane, causing local disturbance, permeability changes, osmotic imbalance, and death cell. The resulting increase in permeability would facilitate the uptake of C/T.

The above-described observations varied from the *in vitro* pharmacodynamics model. We chose three isolates from the most prevalent high-risk clone in our environment, ST175, which had C/T MIC values of between 2 and 16 mg/liter. Interestingly, the human-simulated free concentration of C/T and colistin had a synergistic effect in the ST175 (09-012) isolate, with a C/T MIC of 8 mg/liter. An additive effect was observed in the C/T-susceptible strain ST175 (10-023) and, interestingly, also in the resistant strain ST175 (07-016), with a C/T MIC of 16 mg/liter. It should be noted that the resistant isolate responded to C/T monotherapy, achieving a 3.5-log reduction, which is at odds with data from Rico Caballero et al. (24). Nevertheless, previous studies have demonstrated that adequate C/T concentrations confer a more favorable PTA profile for infections with higher MICs (15, 29, 30).

No antagonism for C/T and colistin against any of the *P. aeruginosa* isolates was observed in our study.

In agreement with previous reports, we have shown that the combined use of β-lactams and colistin led to increased activity against MRD/XDR *P. aeruginosa* compared with that of either agent used as monotherapy. One particularly relevant discovery was the role of this combination in C/T-resistant strains.

In our study, monotherapy with colistin resulted in the development of colistin-resistant subpopulations, and it was also present in the control without any exposure to antibiotic during the experiment. These data are helpful to understand the results, since heteroresistance already could be present, and therefore these isolates killed with the addition of C/T, or resistance could be caused by suboptimal colistin concentrations, and therefore C/T can prevent this resistance development from occurring.

Some limitations of this study should be noted. *In vitro* studies cannot examine toxicity, the contribution of the immune system, or the different PK/PD effects occurring at the specific site of an infection. Studies were only 24 h in length; longer-duration

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experiments are needed to represent the clinical administration guidelines and to assess the emergence of resistance.

To our knowledge, this is the first study to assess the synergy of C/T plus colistin against a large collection of representative XDR *P. aeruginosa* isolates, including prevalent high-risk clones, and the first to focus on ST175 C/T nonsusceptibility isolates. Our findings may help to identify novel strategies to improve the treatment of MDR/XDR *P. aeruginosa* infections using currently available drugs.

MATERIALS AND METHODS

Bacterial isolates. Twenty-four clinical XDR *P. aeruginosa* isolates were used. These isolates previously had been collected from nine Spanish hospitals in the multicenter COLIMERO trial and characterized at a molecular level using pulsed-field gel electrophoresis, multilocus sequence typing, and whole-genome sequencing (26). The selected isolates are representative of all the clones and resistance mechanisms detected in the trial.

Antibiotics. C/T (Zerbaxa) and colistin (colistin sulfate) were provided by Merck Sharp & Dohme (MSD) and from Sigma-Aldrich, respectively. Antibiotic solutions were prepared according to CLSI guidelines (31). Concentrations for time-kill experiments were based on AUC serum levels: for colistin, 4.5 MIU q12h, area under the concentration-time curve for 24 h (AUC_{24}), 50 μg·h/ml (32–34); for C/T, 2/1 g q8h, AUC_{24}, 912/150 μg·h/ml (35).

In the chemostat model, they were administered to simulate free plasma concentrations in critically ill patients under treatment for several infections. The simulated C/T dosing regimen was 2/1 g every 8 h by intravenous infusion over 1 h (current standard) to achieve a free maximum concentration of 90 mg/liter, with a simulated elimination half-life of 3 h and protein binding of 20% (23, 36, 37). It was assumed that tazobactam would be eliminated at the same rate as ceftolozane, since it has a limited role in this drug's activity against *P. aeruginosa* (38). In the dynamic model, we simulated a continuous infusion of colistin to achieve concentrations of 2 mg/liter to mimic plasma colistin concentration-time profiles in critically ill patients (34). Due to protein binding for colistin being 50% (24), we simulated a free steady-state concentration of 1 mg/liter. C/T and colistin concentrations were validated by high-performance liquid chromatography (HPLC) (39, 40).

***In vitro* antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed according to the CLSI guidelines (31) for broth microdilution using cation-adjusted Mueller-Hinton broth (CAMHB).

Time-kill experiments. Time-kill analyses were performed on each of the 24 isolates to analyze the activity of C/T and colistin alone and in combination at clinically achievable free drug concentrations (when maximum indicated clinical doses were used). All experiments were performed in duplicate. The study flow is shown in Fig. 3.

An overnight culture of each isolate was diluted with CAMHB and further incubated at 35°C to reach early-log-phase growth. The bacterial suspension was diluted with CAMHB according to absorbance at 630 nm; 30 ml CAMHB was placed in 50-ml sterile conical flasks with the corresponding antibiotics. The final concentration of the bacterial suspension in each flask was approximately 7 to 8 $\log_{10}$ CFU/ml. Flasks were incubated at 35°C in a shaker water bath for 24 h. Bacterial growth was measured at 0, 2, 4, 8, 12, and 24 h. A 1-ml aliquot was obtained from each flask, centrifuged twice at 5,000 rpm for 5 min, and then reconstituted with sterile saline solution to its original volume to minimize drug carryover. Serial decimal dilutions in CAMHB were performed, and 200 μl was plated on Muller-Hinton E (MHE) agar plates to quantify the total bacterial count for each sample. The inoculated plates were incubated in a humidified incubator (35°C) for 18 to 24 h. Bacterial colonies were counted after the overnight incubation, and the bacterial density from the original sample was calculated based on the dilution factor.

Pharmacodynamic time-kill parameters. Bactericidal effect was defined as a $\geq$3-$\log_{10}$ CFU/ml reduction at 24 h from the starting point of the curve; synergy was defined as a $\geq$2-$\log_{10}$ CFU/ml reduction in the culture at 24 h for the combination compared with the most active single drug; indifference was defined as a $<$2-$\log_{10}$ CFU/ml change at 24 h; antagonism was defined as $\geq$1-$\log_{10}$ CFU/ml regrowth achieved with the combination compared with the least active component; and additivity was defined as a 1- to 2-$\log_{10}$ CFU/ml reduction based on the final count of colonies in the antibiotic combination compared with the count for the more effective of the two components (21, 41).

***In vitro* pharmacodynamic model.** A one-compartment *in vitro* chemostat model (42) was used to validate the C/T plus colistin combination against three isolates of XDR *P. aeruginosa* ST175 with different susceptibility levels to C/T, ST175 (10-023), ST175 (09-012), and ST175 (07-016), which have MICs between 2 and 16 mg/liter. These isolates were selected because ST175 is the most prevalent high-risk clone in our environment. The chemostat model consisted of four independent glassware reactor models studied simultaneously: one contained an antibiotic-free growth control, one contained C/T, one contained colistin, and one contained C/T plus colistin. The experiment was placed in an incubator at 37°C. All reactors were filled with 300 ml of CAMHB broth under constant stirring. Several colonies were inoculated in the reactors to achieve 10^7 to 10^8 $\log_{10}$ CFU/ml of each isolate. They were supplemented with the corresponding concentration of the selected antibiotics, which were infused into the reactors via antibiotic pumps. Antibiotics were added as boluses into the treatment models to achieve target peak concentrations. Fresh broth was supplied via a peristaltic pump (Masterflex L/S model 7524-40; Cole-Parmer Instrument Company, Vernon Hills, IL) programmed to achieve the human-simulated half-life of the antimicrobial being tested. Samples were obtained from each of the models at specific time points

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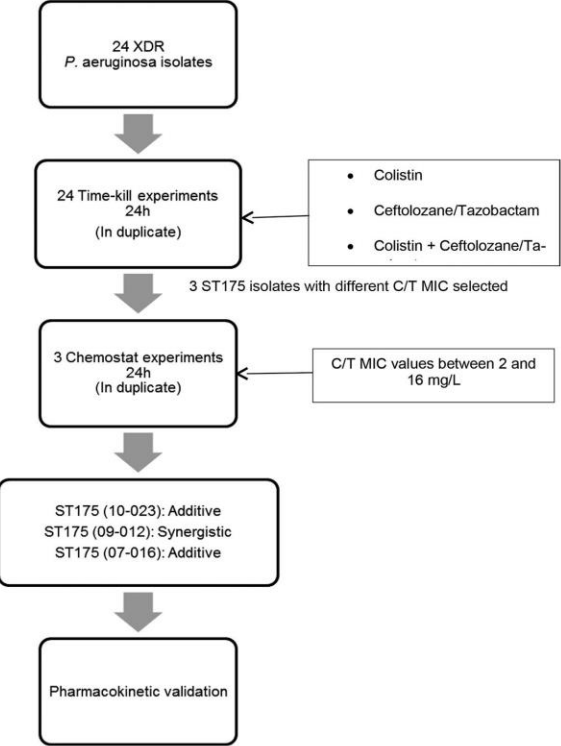

FIG 3 Study flow. We conducted 24 time-kill experiments with three selected antibiotics on our entire collection of *P. aeruginosa* high-risk clones to identify additive and synergistic effects. The combination of C/T plus colistin was validated in three selected ST175 isolates with different C/T susceptibility levels in a one-compartment chemostat model.

(0, 2, 4, 8, 12, and 24 h) throughout the experiment and were serially diluted in normal saline to assess changes in bacterial density over time. Aliquots from each diluted sample were plated onto TSA II plates and incubated at 37°C for 18 to 24 h for quantitative cultures. Numbers of CFU were counted after the overnight incubation. The lower limit of detection was 1.3 $\log_{10}$ CFU/ml. All experiments were conducted in duplicate over 24 h (24).

In the chemostat model, a portion of the bacterial suspension was quantitatively cultured onto agar supplemented with C/T at 2-fold and 4-fold the baseline MIC or with colistin at 2-fold and 4-fold the MIC to assess the effect of each regimen on the less susceptible bacterial population.

Pharmacokinetic studies. Antibiotic concentrations were collected from the reactors at the predetermined time points and stored at −80°C until analysis. Samples were taken to validate antibiotic concentrations. All exposures to simulate the steady-state human pharmacokinetics of unbound drugs were based on half-lives of 3 h for ceftolozane and colistin (43). All treatment regimens were compared with a no-treatment control. Over the first 24 h of the study, all pharmacokinetic samples were determined by HPLC.

Statistical analysis. We performed the nonparametric Kruskal-Wallis test to determine if these differences were statistically significant between the conditions (A, control; B, colistin; C, C/T; D, C/T plus colisitn). This test is preferred to the ANOVA (analysis of variance) test when a normal distribution is not assumed. We corrected multiple comparisons between pairs of variables with Bonferroni correction. A *P* value of ≤0.05 was considered statistically significant.

For antibiotic exposures, we performed a linear regression and assessed the global fit with the coefficient of determination (R^2), which represents the proportion of variability of the dependent variable (*Y*) that can be attributed to *X*.

For each regimen (including the growth control) for the duration of the study, the LR difference in area under the curve for CFU (AUCFU) was calculated to compare any two regimens (test and reference). We compared each antibiotic alone with the control isolate and each antibiotic combination with the

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antibiotic alone. We calculated the log ratio of AUCFU (LR) as LR = $\log_{10}$ ($AUCFU_{test}/AUCFU_{reference}$), where the reference regimen is the growth control. An LR value of −1 or −2 means that the test regimen (compared to the reference) reduced exposure by 90% (10-fold reduction) or 99% (100-fold reduction), respectively. No definitions for synergy, additivity, etc., have been established for the LR of the AUCFU method (24, 44).

ACKNOWLEDGMENTS

We thank the Institute for Clinical Pharmacodynamics (ICPD), Schenectady, NY, and the Infectious Pathology and Antimicrobials Research Group (IPAR), Institute Hospital del Mar d' Investigacions Mèdiques (IMIM), for their support.

This study was partially supported by the Ministerio de Economía y Competitividad of Spain, Instituto de Salud Carlos III.FEDER (PI16/00669 and PI17-00251).

We have no conflicts of interest to declare.

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4.3. Time-kill evaluation of antibiotic combinations containing ceftazidime-avibactam against extensively drug-resistant *Pseudomonas aeruginosa* and their potential role against ceftazidime-avibactam-resistant isolates.

Microbiology Spectrum 2021; 9(1): e0058521.
DOI: 10.1128/Spectrum.00585-21.

RESEARCH ARTICLE

Time-Kill Evaluation of Antibiotic Combinations Containing Ceftazidime-Avibactam against Extensively Drug-Resistant *Pseudomonas aeruginosa* and Their Potential Role against Ceftazidime-Avibactam-Resistant Isolates

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ABSTRACT Ceftazidime-avibactam (CZA) has emerged as a promising solution to the lack of new antibiotics against *Pseudomonas aeruginosa* infections. Data from *in vitro* assays of CZA combinations, however, are scarce. The objective of our study was to perform a time-kill analysis of the effectiveness of CZA alone and in combination with other antibiotics against a collection of extensively drug-resistant (XDR) *P. aeruginosa* isolates. Twenty-one previously characterized representative XDR *P. aeruginosa* isolates were selected. Antibiotic susceptibility was tested by broth microdilution, and results were interpreted using CLSI criteria. The time-kill experiments were performed in duplicate for each isolate. Antibiotics were tested at clinically achievable free-drug concentrations. Different treatment options, including CZA alone and combined with amikacin, aztreonam, meropenem, and colistin, were evaluated to identify the most effective combinations. Seven isolates were resistant to CZA (MIC $\geq$ 16/4 mg/liter), including four metallo-β-lactamase (MBL)-carrying isolates and two class A carbapenemases. Five of them were resistant or intermediate to aztreonam (MIC $\geq$ 16 mg/liter). Three isolates were resistant to amikacin (MIC $\geq$ 64 mg/liter) and one to colistin (MIC $\geq$ 4 mg/liter). CZA monotherapy had a bactericidal effect in 100% (14/14) of the CZA-susceptible isolates. Combination therapies achieved a greater overall reduction in bacterial load than monotherapy for the CZA-resistant isolates. CZA plus colistin was additive or synergistic in 100% (7/7) of the CZA-resistant isolates, while CZA plus amikacin and CZA plus aztreonam were additive or synergistic in 85%. CZA combined with colistin, amikacin, or aztreonam was more effective than monotherapy against XDR *P. aeruginosa* isolates. A CZA combination could be useful for treating XDR *P. aeruginosa* infections, including those caused by CZA-resistant isolates.

IMPORTANCE The emergence of resistance to antibiotics is a serious public health problem worldwide and can be a cause of mortality. For this reason, antibiotic treatment is compromised, and we have few therapeutic options to treat infections. The main goal of our study is to search for new treatment options for infections caused by difficult-to-treat resistant germs. *Pseudomonas aeruginosa* is a Gram-negative bacterium distributed throughout the world with the ability to become resistant to most available antibiotics. Ceftazidime-avibactam (CZA) emerged as a promising solution to the lack of new antibiotics against infections caused by *P. aeruginosa*

Citation Montero MM, Domene Ochoa S, López-Causapé C, Luque S, Sorlí L, Campillo N, López Montesinos I, Padilla E, Prim N, Angulo-Brunet A, Grau S, Oliver A, Horcajada JP. 2021. Time-kill evaluation of antibiotic combinations containing ceftazidime-avibactam against extensively drug-resistant *Pseudomonas aeruginosa* and their potential role against ceftazidime-avibactam-resistant isolates. Microbiol Spectr 9:e00585-21. https://doi.org/10.1128/Spectrum.00585-21.

Editor William Lainhart, University of Arizona/Banner Health

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Received 18 June 2021
Accepted 29 June 2021
Published 28 July 2021

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strains. This study intended to analyze the effect of CZA alone or in combination with other available antibiotics against *P. aeruginosa* strains. The combination of CZA with other antibiotics could be more effective than monotherapy against extensively drug-resistant *P. aeruginosa* strains.

KEYWORDS ceftazidime-avibactam, colistin, aztreonam, amikacin, combination therapy, *Pseudomonas aeruginosa*

New therapeutic options for multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* infections are required to overcome the growing problem of antimicrobial resistance. According to the U.S. Centers for Disease Control and Prevention, XDR *P. aeruginosa* is a "serious threat" to human health, and resistance is on the rise (1). This bacterium has a nonclonal epidemic population structure (2) and can develop antibiotic resistance through several mechanisms. XDR *P. aeruginosa* high-risk clones are disseminated in hospitals around the world (2) and pose a major public health problem because of limited treatment options and rising costs. Sequence type 111 (ST111) and ST235 are the predominant high-risk clones worldwide, but in Spain, the predominant clone is ST175 (2). High-risk clones are frequently responsible for nosocomial infections and are associated with the acquisition of horizontally transferable beta-lactamases and resistance mechanisms through chromosomal mutations (2, 3).

The problem of increasing antimicrobial resistance is compounded by a dwindling supply of new drugs. Given the few antibiotics in the clinical pipeline before 2010, the treatment options for XDR *P. aeruginosa* infections were suboptimal and consisted largely of antibiotics with a narrow therapeutic window and high toxicity (aminoglycosides, polymyxins) or unpredictable pharmacokinetics (colistin), yielding poor patient outcomes (4–7).

Ceftazidime-avibactam (CZA) was approved by the U.S. Food and Drug Administration in 2015 and was the first β-lactam combination to provide broad coverage against XDR Gram-negative pathogens, including *P. aeruginosa* (8). Few studies, however, have examined the effectiveness of CZA against infections caused by XDR *P. aeruginosa* high-risk clones. An *in vitro* study of a large collection of *P. aeruginosa* strains reported a CZA resistance rate of 2.9% (9). Most studies, however, have reported higher rates, up to 18% in some cases (10) and over 50% when XDR strains are involved (11, 12). Strains carrying metallo-β-lactamases (MBLs) have the highest resistance rates (>95%) as they are resistant to CZA, and CZA is not expected to be efficacious against these strains (13).

The use of CZA to treat *P. aeruginosa* infections caused by XDR high-risk clones may be clinically more effective and less toxic than colistin, which is often the only option available (14). However, given the high risk for the emergence of CZA-resistant mutants, it is paramount to monitor their selection during treatment and to evaluate associated risk factors. Combination therapy is a useful strategy for achieving maximum antimicrobial activity against various resistant organisms and for preventing antibiotic resistance (15). *In vitro* experiments have shown synergy for certain antipseudomonal antibiotics against MDR *P. aeruginosa* (5, 15–20). *In vitro* studies evaluating the activity of CZA combined with other antibiotics against *P. aeruginosa*, however, are lacking, and only few reports covering a small number of isolates have been published (21).

The aim of this study was to perform a comprehensive time-kill analysis of CZA alone or in combination with standard antipseudomonal antibiotics against a representative collection of the most common resistance mechanisms and XDR *P. aeruginosa* clones, including high-risk clones.

RESULTS

Antimicrobial susceptibility testing. The antibiotic susceptibility profiles and previously characterized antibiotic resistance mechanisms of the 21 XDR *P. aeruginosa* isolates are shown in Table 1. Seven isolates were resistant to both CZA (MIC $\geq$ 16/4 mg/liter) and meropenem (MIC $\geq$ 8 mg/liter), and of these, four were resistant and one was intermediate

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TABLE 1 Antibiotic susceptibility profile and resistance mechanisms of the 21 XDR *P. aeruginosa* isolates[a]

					MIC (mg/liter)				
Isolate	**ST**	**Acquired β- lactamase(s)**	**AmpC hyperproduction**	**OprD deficiency**	**AMK**	**ATM**	**MEM**	**CST**	**CZA**
04-017	111	OXA-46	Yes	No	4	64	32	2	8
04-025	175		Yes	Yes	4	16	16	1	4
10-023	175		Yes	Yes	4	16	16	2	4
06-014	179	OXA-10	Yes	Yes	8	16	32	2	4
12-003	244		Yes	Yes	8	32	32	2	4
09-011	274		Yes	Yes	128	64	32	1	4
09-007	313		Yes	Yes	8	32	16	2	4
10-017	395		Yes	No	4	32	8	2	4
06-035	455		Yes	No	<2	64	>32	0.5	8
10-019	2221		Yes	Yes	<2	64	32	2	8
06-025	2534		Yes	Yes	<2	64	8	2	8
06-027	2535		Yes	No	8	32	8	2	4
06-001	2536		Yes	Yes	8	64	32	2	8
09-012	175		Yes	Yes	8	64	16	2	8
10-009	111	VIM-2	Yes	Yes	32	>128	>32	4	>32
07-016	175	GES-5	No	Yes	16	16	>32	2	32
12-012	175	VIM-20, OXA-2	No	Yes	16	8	>32	2	32
07-004	235	GES-19, OXA-2	No	Yes	128	128	>32	2	>32
06-042	235	VIM-47	No	No	64	32	>32	2	32
01-008	253	VIM-1	No	Yes	8	4	>32	2	>32
10-021	2533		Yes	Yes	<2	64	32	1	16

[a]MICs (mg/liter) of the following antibiotics tested in this study are shown: amikacin (AMK), aztreonam (ATM), meropenem (MEM),colistin (CST), and ceftazidime-avibactam (CZA). CZA-resistant isolates are highlighted in gray.

to aztreonam (MIC ≥ 16 mg/liter), three were resistant to amikacin (MIC ≥ 64 mg/liter), and one was resistant to colistin (MIC ≥ 4 mg/liter). Six of the seven CZA-resistant isolates harbored carbapenemases belonging to Ambler class A or B and had OprD deficiency, except for one, and two of them showed AmpC hyperproduction.

Time-kill studies. Bacterial growth without antibiotic reached 8 to 9 log_{10} CFU/ml at 24 h for all isolates. The results of the time-kill experiments for the 21 XDR *P. aeruginosa* isolates are shown in Table S1 in the supplemental material. The mean bacterial loads (log_{10} CFU/ml) over 24 h for the seven CZA-resistant XDR *P. aeruginosa* isolates treated with each antibiotic regimen are shown in Fig. 1. Table 2 shows the synergistic and additive effects of each combination against CZA-susceptible and CZA-resistant isolates. Table S2 shows the time-kill results (log difference at 24 h) for each antibiotic compared with the control and for each antibiotic combination compared with each antibiotic.

Single antibiotics (aztreonam, meropenem, colistin, amikacin) were not bactericidal against any of the isolates at 24 h. Despite this, when compared with the control, all single antibiotics resulted in fewer bacteria than the control ($F_{4, 64} = 8.7$, $P < 0.001$; amikacin dif = −1.34, $t = -2.5$, $P = 0.02$; aztreonam dif = −1.43, $t = -2.63$, $P = 0.01$; meropenem dif = −1.42, $t = -2.62$, $P = 0.01$; colistin dif = −3.18, $t = -5.87$, $P < 0.001$).

CZA monotherapy was bactericidal against all the CZA-susceptible isolates, with a mean reduction of 3.19 log_{10} CFU/ml. In a comparison of the effects of the combination of CZA with other antibiotics, we found differences ($F_{4, 65} = 11.08$, $P < 0.001$). CZA plus amikacin (dif = −1.74, $t = -3.58$, $P < 0.001$) and CZA plus colistin (dif = −1.59, $t = -3.25$, $P = 0.001$) achieved a mean reduction of >4 log_{10} CFU/ml in the same isolates. The best combination against the CZA-susceptible isolates was CZA plus amikacin, which was synergistic or additive in approximately 80% of cases. On the other hand, no differences between CZA alone and CZA with aztreonam were found (dif = −0.48, $t = -0.99$, $P = 0.33$). Furthermore, combining CZA with meropenem increased the number of bacteria in comparison with CZA alone (dif = 1.02, $t = 1.09$, $P = 0.04$).

CZA combination therapies achieved a higher overall reduction in bacterial load than any of the treatments in isolation for the seven CZA-resistant isolates ($F_{1, 61} = 33.92$, $P < 0.001$). The log_{10} CFU/ml mean for the treatments in isolation was 0.94, and combining

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FIG 1 Bacterial load (log_{10} CFU/ml) over 24 h in the seven CZA-resistant XDR *P. aeruginosa* isolates for each antibiotic regimen. LOD, lower limit of detection.

treatments reduced that mean to 3.44 ($t = -5.82$, $P < 0.001$). Hence, the mean reduction was 4.4 log_{10} CFU/ml for CZA plus colistin, amikacin, or aztreonam. As can be seen in Table 2, CZA plus colistin was either additive or synergistic in 100% of cases, while CZA plus amikacin or aztreonam was additive or synergistic in 85% of cases. The combination of CZA with aztreonam was effective against three of the four MBL-carrying isolates and against the two isolates that harbored class A carbapenemases.

DISCUSSION

We investigated the use of CZA alone or in combination with four antibiotics to assess the potential synergistic effects against XDR *P. aeruginosa*. As expected, a bactericidal effect was observed for CZA monotherapy in all the CZA-susceptible *P. aeruginosa* isolates, which had AmpC hyperproduction and/or OprD deficiency. To preserve the effectiveness of CZA, its clinical use should be avoided in naturally resistant strains and in those carrying MBLs and certain class D β-carbapenemases (22). Combination

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TABLE 2 Synergistic and additive effects of each antibiotic combination against CZA-susceptible and CZA-resistant *P. aeruginosa* isolates[a]

	% of isolates					
	CZA susceptible			CZA resistant		
Antibiotic combination	Synergy	Additivity	Total	Synergy	Additivity	Total
AMK+CZA	8	3	78.6	5	1	85.7
ATM+CZA	2	4	42.9	4	2	85.7
MEM+CZA	1	1	14.3	2	0	28.6
CST+CZA	6	2	57.1	6	1	100.0

[a]AMK, amikacin; ATM, aztreonam; MEM, meropenem; CST, colistin; CZA, ceftazidime-avibactam.

therapy has an important role in these clinical scenarios, and CZA combined with other antibacterial agents should be considered.

CZA resistance has already been described in Gram-negative bacilli. β-Lactamase-related mutations are the main mechanism behind CZA resistance in *Enterobacterales*. Recent reports suggest that the development of different resistance mechanisms within the course of treatment (e.g., mutations in KPC-encoding genes) might threaten the effectiveness of CZA (23, 24), a phenomenon that could be further complicated by horizontal spread (25). The development of CZA resistance during treatment of *P. aeruginosa* infections is frequently due to the selection of mutations in the AmpC β-lactamase structure, which are associated with coresistance with ceftolozane-tazobactam (16). Other contributory factors might be diminished outer membrane permeability and/or overexpression of efflux pumps (26). High-level resistance to CZA might also be due to MBL acquisition (27). Overall, six of the seven CZA-resistant isolates in our study harbored acquired β-lactamases, including several MBLs (VIM type) and a serine carbapenemase.

Little has been published on antibiotic combinations containing CZA, especially in the context of XDR *P. aeruginosa* isolates. Combination therapy with CZA plus aztreonam, amikacin, colistin, fosfomycin, and meropenem was recently evaluated in MDR *Klebsiella pneumoniae and P. aeruginosa* strains, but none of the isolates carried MBLs and few time-kill curves were analyzed (28). A synergistic effect was also reported for the combined use of CZA and colistin against MDR *P. aeruginosa* strains, including those resistant to colistin (29). In the present study, the combination of CZA with colistin showed a synergistic or additive effect against all the CZA-resistant *P. aeruginosa* isolates, including a colistin-resistant strain. Synergy was also observed against 85% of these isolates when CZA was combined with amikacin or aztreonam. In the combination of CZA with colistin, several bacterial isolates reached bacterial eradication at 4 and 8 h but then showed a little regrowth at 24 h. The phenomenon of bacterial regrowth could be due to either a loss of functionality of these antibiotics or selection of resistant isolates. Presumably, the latter could include selection of preexisting resistant subpopulations, *de novo* mutations, adaptive resistance, or formation of persistent cells (30). Further studies are required in order to evaluate these possibilities.

A double β-lactam strategy has been tested against carbapenemase-producing enterobacterial isolates in which CZA combined with meropenem or imipenem showed synergy against certain KPC-producing *K. pneumoniae* strains (31). In our study, however, CZA plus meropenem was the only combination to show no synergistic or additive activity against most XDR *P. aeruginosa* isolates. This could be because nonenzymatic mechanisms, alongside acquired β-lactamases, may have contributed to high meropenem MICs in the CZA-resistant isolates.

As mentioned, CZA is not active against MBL-bearing strains (22). The addition of aztreonam might overcome this resistance, as MBLs are known to have a weak hydrolysis capacity against aztreonam (32, 33). Combination therapy with ceftazidime and aztreonam may also be beneficial due to the simultaneous inhibition of multiple

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penicillin-binding proteins (34). Additionally, CZA plus aztreonam could exert an independent effect by acting on the "divisome" of Gram-negative bacteria (27). A recent report based on time-kill experiments with five *P. aeruginosa* isolates resistant to both CZA and aztreonam found that the combined use of the antibiotics had a synergistic effect and restored bactericidal activity in four of the isolates (21). In our study, this combination was effective against three of the four MBL-carrying isolates.

This study had some limitations. Our results are based on short *in vitro* assays with minimal antibiotic exposure compared with other pharmacokinetic/pharmacodynamic studies. Since these results are not representative of clinical guidelines for the administration of most antibiotics, they must be validated in *in vivo* experiments (35). The experimental design of this type of study does not allow identification of mechanisms of interactions or taking the emergence of resistance into consideration. A strength of our study is that our results are based on a large number of time-kill assays and show evidence of synergistic or additive effects in a considerable proportion of cases.

In conclusion, CZA is effective against XDR *P. aeruginosa* isolates both alone and in combination with other antibiotics. Combination regimens featuring CZA may be a good option against infections caused by these difficult-to-treat bacteria. Our data support the potential use of CZA in combination with amikacin, aztreonam, and colistin against XDR *P. aeruginosa* isolates, including CZA-resistant isolates and prevalent high-risk clones. These findings may help identify strategies to improve the clinical management of XDR *P. aeruginosa* infections using currently available drugs.

MATERIALS AND METHODS

Bacterial isolates and resistance mechanisms. We studied 21 XDR *P. aeruginosa* clinical isolates which had been previously collected by our group as a part of the COLIMERO trial, a multicenter Spanish trial involving the molecular characterization of 150 XDR *P. aeruginosa* isolates from nine Spanish hospitals using pulsed-field gel electrophoresis, multilocus sequence typing, and whole-genome sequencing (3). The 21 isolates were representative of the clones and the most prevalent and relevant resistance mechanisms detected in the trial, namely, chromosomal mutations (AmpC hyperproduction and OprD inactivation) and horizontally acquired enzymes, including several MBLs and class A carbapenemases.

Antibiotics. The antipseudomonal antibiotics used in the experiments were amikacin, aztreonam, colistin, meropenem (Sigma-Aldrich), and CZA (Pfizer). The antibiotics were chosen based on the mechanism of action and availability in the hospital's pharmacy. Antibiotic solutions were prepared according to CLSI guidelines (36). Antibiotic concentrations for time-kill experiments were based on area-under-the-curve (AUC) serum levels: for amikacin, 1 g every 24 h (q24h), with an area under the concentration-time curve for 24 h (AUC_{24}) of 196 μg · h/ml (37, 38); for aztreonam, 2 g q8h, with an AUC_{24} of 1,050 μg · h/ml (39); for meropenem, 2 g q8h, with an AUC_{24} of 425 μg · h/ml (40); for colistin, 4.5 MIU (million International units) q12h, with an AUC_{24} of 50 μg · h/ml (41, 42); for CZA, 2 g q8h, with an AUC_{24} of 800 μg · h/ml (43); and for avibactam, 2 g q8h, with an AUC_{24} of 147 μg · h/ml (43).

Antibiotic susceptibility testing. The susceptibility profiles of the XDR isolates were obtained from the COLIMERO trial (3). Antimicrobial susceptibility was tested using broth microdilution and agar dilution methods with cation-adjusted Mueller-Hinton II broth (CAMHB) and Mueller-Hinton (MH) agar media, according to the CLSI guidelines (36). Ceftazidime susceptibility testing was conducted alone and in combination with a fixed avibactam concentration (4 mg/liter).

Time-kill experiments. Time-kill studies were performed to analyze the activity of the selected antibiotics alone and in combination with CZA at clinically achievable free-drug concentrations. All experiments were performed in duplicate. An overnight culture of isolate was diluted with CAMHB and further incubated at 37°C for an hour to reach early log-phase growth. The bacterial suspension was diluted with CAMHB according to the absorbance at 630 nm. The magnitudes of absorbance ranged from 0.2 to 0.4. Sterile 50-ml conical flasks were used with 30 ml of CAMHB supplemented with the corresponding antibiotics. The final bacterial inoculum was approximately 6 to 7 $\log_{10}$ CFU/ml per flask. Flasks were incubated at 37°C in a shaker water bath for 24 h. Samples were collected at 0, 4, 8, and 24 h to measure bacterial growth. A 1-ml aliquot was obtained from each flask at each time point, centrifuged at 13,000 rpm for 3 min, and reconstituted with sterile saline solution to its original volume to minimize drug carryover. Serial decimal dilutions in CAMHB were performed; MH agar plates were inoculated (200 μl per plate) and incubated in a humidified incubator (37°C) for 18 to 24 h. Bacterial colonies for each sample were counted after overnight incubation. The bacterial density from the original sample was calculated based on the dilution factor. The limit of detection (LOD) was 1.3 $\log_{10}$ CFU/ml.

Apart from describing the results, in order to assess the effect of monotherapy and of the antibiotic combinations, we performed a series of regression analyses in which we entered the log difference in 24 h as dependent variable and each antibiotic regimen as independent variable. We checked for the application conditions of the regression, and all the conditions were met (normality of the residuals [assessed with Shapiro-Wilk's test] and homoscedasticity [assessed with the Breusch-Pagan test]).

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Pharmacodynamic time-kill parameters. The results of the time-kill experiments were read at the different time points (0, 4, 8, and 24 h). Bactericidal activity was defined as a $\geq$3-log_{10} CFU/ml reduction, synergy as a $\geq$2-log_{10} CFU/ml reduction for a given combination compared with the most active single agent, additivity as a 1- to 2-log_{10} CFU/ml reduction in the final colony count for the combination compared with the most active single agent, and antagonism as a regrowth to $\geq$1-log_{10} CFU/ml for the combination compared with the least active single agent (44, 45). In addition to the aforementioned relevance criteria, we applied regression analysis to determine if the difference in log_{10} was statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank the Institute for Clinical Pharmacodynamics (ICPD), Schenectady, NY, and the Infectious Pathology and Antimicrobials Research Group (IPAR), Institute Hospital del Mar d'Investigacions Mèdiques (IMIM), for their support.

This study was supported by a medical grant from Pfizer Spain and was partially supported by the Ministerio de Economía y Competitividad of Spain, Instituto de Salud Carlos III, FEDER PI16/00669, PI17/00251, and PI18/0076, and the Spanish Network for Research in Infectious Diseases (REIPI RD16/0016).

We declare no conflicts of interest.

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4.4. Impact of ceftolozane-tazobactam concentrations in continuous infusion against extensively drug-resistant *Pseudomonas aeruginosa* isolates in a hollow-fiber infection model.

Scientific Reports 2021; 11(1): 22178.
DOI: 10.1038/s41598-021-01784-4.

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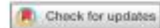

OPEN

Impact of ceftolozane/tazobactam concentrations in continuous infusion against extensively drug-resistant *Pseudomonas aeruginosa* isolates in a hollow-fiber infection model

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Ceftolozane/tazobactam (C/T) has emerged as a potential agent for the treatment of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* infections. As it is a time-dependent antimicrobial, prolonged infusion may help achieve pharmacokinetic/pharmacodynamic (PK/PD) targets. To compare alternative steady-state concentrations (Css) of C/T in continuous infusion (CI) against three XDR *P. aeruginosa* ST175 isolates with C/T minimum inhibitory concentration (MIC) values of 2 to 16 mg/L in a hollow-fiber infection model (HFIM). Duplicate 10-day HFIM assays were performed to evaluate Css of C/T in CI: one compared 20 and 45 mg/L against the C/T-susceptible isolate while the other compared 45 and 80 mg/L against the two C/T-non-susceptible isolates. C/T resistance emerged when C/T-susceptible isolate was treated with C/T in CI at a Css of 20 mg/L; which showed a deletion in the gene encoding AmpC β-lactamase. The higher dosing regimen (80 mg/L) showed a slight advantage in effectiveness. The higher dosing regimen has the greatest bactericidal effect, regardless of C/T MIC. Exposure to the suboptimal Css of 20 mg/L led to the emergence of C/T resistance in the susceptible isolate. Antimicrobial regimens should be optimized through C/T levels monitoring and dose adjustments to improve clinical management.

The indiscriminate use of antibiotics has contributed to the emergence and selection of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria[1,2] and led to a critical decrease in the availability of alternative antibiotic treatments, limiting treatment options and increasing morbidity and mortality[3]. *Pseudomonas aeruginosa* has an outstanding capacity to develop resistance through a broad range of mechanisms[4–6]. MDR/XDR *P. aeruginosa* isolates are particularly concerning, as they are the leading cause of nosocomial infections and a strong contributor to in-hospital mortality[7]. The ST175 clone is especially significant in several European countries[8]. The development of tailored antimicrobial treatments could greatly improve the clinical management of infections caused by MDR/XDR *P. aeruginosa*.

Ceftolozane/tazobactam (C/T; Zerbaxa; Merck & Co, Inc., Kenilworth, NJ) has emerged as a potential agent against MDR/XDR strains that are resistant to all first-line antibiotics[9]. The combination of ceftolozane, a cephalosporin, and tazobactam, a beta-lactamase inhibitor[6], has shown promising results in the treatment of infections caused by *P. aeruginosa* strains with different resistance patterns[10]. The current recommended dosage for C/T is

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a 1-h infusion of 1/0.5 g every 8 h for urinary tract and soft tissue infections and 2/1 g every 8 h for respiratory infections[11]. The pharmacokinetic (PK) properties of ceftolozane have been studied alone and combined with tazobactam in healthy individuals[6,12]. As C/T is a time-dependent antibiotic, the percentage of time during the dosing interval in which free drug plasma concentrations remain above the minimum inhibitory concentration (MIC) (%$T_{>MIC}$) is the best pharmacodynamic (PD) parameter for predicting bacteriological efficacy. The %$T_{>MIC}$ is approximately 40–50% for some cephalosporin[6,12], but recent studies have shown that the percentage for ceftolozane is much lower, similar to that reported for carbapenems[13,14]. The currently recommended C/T dosing regimen thus might be insufficient against *P. aeruginosa* strains with a C/T MIC above the susceptibility breakpoint of 4 mg/L. Infections caused by these strains would therefore need to be treated with combinations of antibiotics or optimized dosing[15].

The aim of this study was to evaluate different steady-state concentrations (Css) of C/T in continuous infusion (CI) to test the effectiveness of C/T and the emergence of resistance in an in vitro hollow-fiber infection model (HFIM). Three XDR *P. aeruginosa* ST175 isolates with different C/T MICs (2, 8 and 16 mg/L) were tested.

Material and methods

Bacterial isolates. Three XDR *P. aeruginosa* clinical isolates were analysed: ST175 (10-023), with a C/T MIC of 2 mg/L; ST175 (09-012), with a C/T MIC of 8 mg/L; and ST175 (07-016), with a C/T MIC of 16 mg/L. These isolates had been previously characterized at a molecular level using pulsed-field gel electrophoresis, multi-locus sequence typing, and whole genome sequencing and are representative of the clones and resistance mechanisms in our environment[16].

Antibiotics. C/T (Zerbaxa®; lot number SO15404; expiration date, August 2020) was provided by Merck & Co., Inc. (Kenilworth, NJ). CI C/T dosing regimens were simulated to achieve approximate Css of 20, 45 and 80 mg/L (which respectively correspond to 3, 6 and > 9 g/4.5 g every 24 h)[17]. The exposures to simulate the steady-state human pharmacokinetics of unbound drug were based on elimination half-life of 3 h for ceftolozane[18,19]. A protein-binding estimate was 20% for ceftolozane. The C/T regimens included a dose range based on previously determined C_{max} and AUC[19]. Exposure to tazobactam was not considered, as this drug has a limited role in ceftolozane's activity against *P. aeruginosa*[20]. C/T concentrations were validated by high-performance liquid chromatography (HPLC)[21].

HFIM. The HFIM has been used extensively and described elsewhere[18,22]. Duplicate 10-day HFIM assays were conducted in two stages to investigate the effectiveness of C/T and the development of antimicrobial resistance. Effectiveness was investigated by treating *P. aeruginosa* isolates ST175 (09-012) and ST175 (07-016) with C/T in CI at steady-state concentrations of 45 and 80 mg/L, while resistance was investigated by treating ST175 (10-023) to C/T in CI at steady-state concentrations of 20 and 45 mg/L.

Polyethersulfone hemofilters where used as the hollow-fiber cartridges (Aquamax HF03, Nikkiso, Belgium). Each C/T regimen was pumped into the corresponding reservoir by a separate infusion pump to simulate human free drug PK profiles in humans. Fresh drug-free growth medium (cation-adjusted Mueller–Hinton broth [CAMHB]) was continuously infused into the central reservoir to dilute and simulate drug elimination in humans. An equal volume of drug-containing medium was concurrently removed from the central reservoir to maintain an isovolumetric system. The extracapillary space of each HFIM was inoculated with 50 mL of bacterial suspension. High-inoculum infections were simulated. Once inoculated, the bacteria were left in the extracapillary compartment of the HFIM cartridge, where they were exposed to fluctuating drug concentrations. The assays were conducted at 37 °C. Maintenance doses were given continuously at the same rate, according to the clinical dosing frequency. Bacterial densities ($\log_{10}$ CFU/mL) in the cartridges were measured at 0, 8, 24, 48, 72, 96, 144, 168, 192 and 240 h. The samples were washed and suspended in saline solution to minimize drug carryover. Serial decimal dilutions were cultured onto drug-free trypticase soy agar (BBL TSA II, Becton Dickinson) plates to determine the total bacterial population. The lower limit of detection (LLOD) was 1.3 $\log_{10}$ CFU/mL. Study flow is shown in Fig. 1. Bactericidal activity was defined as a reduction of 3 $\log_{10}$ CFU/mL from the initial bacterial load[23].

Resistance studies. An aliquot of bacterial suspension from each HFIM was cultured onto drug-containing plates (TSA agar) supplemented with C/T at twofold, fourfold and eightfold the baseline MIC to assess the effect of each regimen on the least susceptible bacterial population. Mutants that grew on these plates were compared with total bacterial population on drug-free TSA plates. When growth was observed after 72 h, up to three colonies were selected to assess C/T MICs and were analysed for changes in MICs from baseline. Antibiotic susceptibility testing was performed according to Clinical & Laboratory Standards Institute (CLSI) guidelines for broth microdilution using CAMHB[24]. The isolates were serially passaged three times on drug-free plates to assess the stability of the phenotype. To investigate the mechanisms leading to C/T resistance, the presence of structural mutations in the catalytic centre of AmpC was analyzed by PCR and sequencing as whole genome sequencing as previously described[25].

Drug concentrations. Antibiotic samples were collected at different time points over the first 48 h (0, 3, 5, 7, 9, 23, 25 27, 29 and 47 h) and once a day for the first dose, until the end of the study. Samples were stored at −80 °C until analysis. All exposures to simulate steady-state human PK of unbound drug were based on the half-life of ceftolozane (exposure to tazobactam was not considered as previously mentioned). Antibiotic concentrations were analysed by HPLC[21].

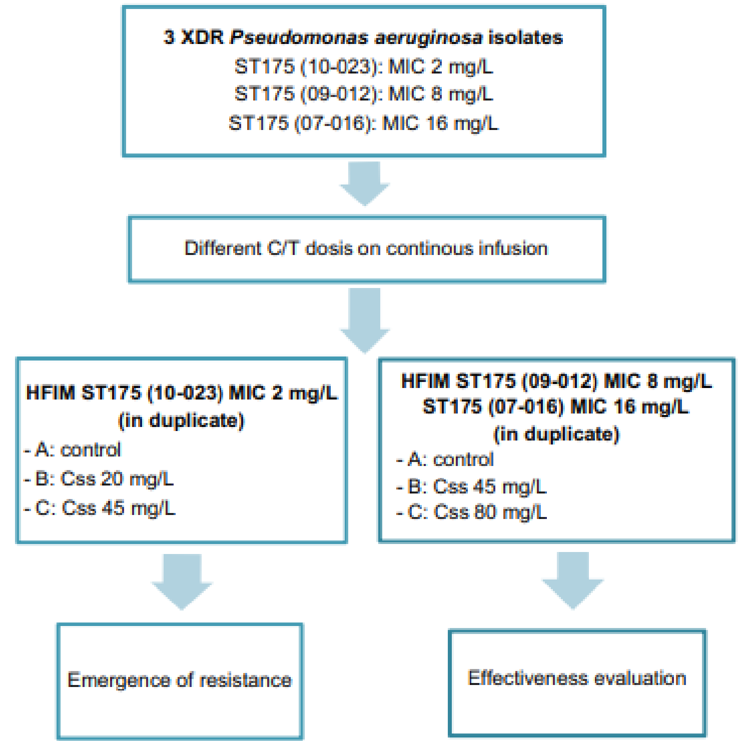

Figure 1. Study flow showing in vitro 10-day HFIM assays conducted with three XDR *Pseudomonas aeruginosa* ST175 isolates with C/T MICs ranging from 2 to 16 mg/L using different Css of C/T in CI: 20 and 45 mg/L to test the emergence of C/T resistance in the susceptible ST175 (10-023) isolate, and 45 and 80 mg/L to test the effectiveness of C/T against the non-susceptible isolates ST175 (09-012) and ST175 (07-016). C/T, ceftolozane/tazobactam; HFIM, hollow-fiber infection model; MIC, minimum inhibitory concentration; XDR, extensively-drug resistant; Css, steady-state concentration; CI, continuous infusion.

Results

In vitro *susceptibility and resistance mechanisms*. The isolates had been previously characterized at a molecular level[16]. The ST175 (10-023) isolate was susceptible to C/T (MIC 2 mg/L) and resistant to the other β-lactams due to OprD inactivation and AmpC hyperproduction[16]. The ST175 (09-012) isolate had intermediate resistance to C/T (MIC 8 mg/L) and the mechanisms identified were OprD inactivation, AmpC hyperproduction, and a mutation in PBP3 (R504C) that has been previously associated with increased β-lactam resistance[16]. The ST175 (07-016) isolate was resistant to C/T (MIC 16 mg/L) and in this case the mechanism identified was the production of a class A carbapenemase GES-5 coupled with OprD inactivation[16].

HFIM and data analysis. Table 1 shows the total mean reduction (log difference at 24 h) for each antibiotic compared with the control. In Fig. 2 results for the reductions in density over time are shown. For ST175 (10-023) the mean bacterial density of the starting inoculum was 7.54 $\log_{10}$ CFU/mL (Fig. 2A). A five $\log_{10}$ CFU/mL reduction was observed for the Css of 45 mg/L. The Css of 20 mg/L was associated with an initial reduction followed by regrowth on day 6. The final bacterial density was 7 ± 0.45 $\log_{10}$ CFU/mL, which corresponds to an overall reduction of 0.54 $\log_{10}$ CFU/mL (no bactericidal effect).

The 10-day HFIM studies to evaluate the effectiveness of higher than the standard Css of C/T (80 mg/L in CI) were performed using the two non-susceptible isolates, ST175 (09-012) and (07-016). The mean starting inoculum was 6.76 $\log_{10}$ CFU/mL. Figure 2B, C shows the changes in bacterial density at the different time points analyzed. Overall, the C/T Css of 80 mg/L in CI showed a slight advantage over the Css of 45 mg/L. Both dosing regimens showed similar effectiveness against ST175 (09-012) up to day 6, but on day 7, the higher regimen achieved eradication of the bacterial population (below the LLOD). The curve for the Css of 45 mg/L plateaued and the final density was 1.65 ± 0.6 $\log_{10}$ CFU/mL. A similar pattern was observed for ST175 (07-016), but in this

	ST175 (10-023)	ST175 (09-012)	ST175 (07-016)
	Log diff day 10[a]	Log diff day 10	Log diff day 10
C/T 3 g q24 h CI Css 20 vs Control	− 2.48 ± 0.14	–	–
C/T 6 g q24 h CI Css 45 vs Control	− 6.94 ± 0.05	− 8.16 ± 0.17	− 7.04 ± 0.06
C/T 9 g q24 h CI Css 80 vs Control	–	− 9.81 ± 0.08	− 7.93 ± 0.10

Table 1. Mean overall reduction in bacterial density ($\log_{10}$ CFU/mL ± standard deviation) using alternative C/T Css regimens for each isolate. *C/T* ceftolozane/tazobactam, *CI* continuous infusion, *Css* steady-state concentration. [a]Log difference at the end of the assay for each regimen compared with the control.

case, the Css of 80 mg/L did not eradicate the bacterial population. In brief, C/T at both Css (45 and 80 mg/L) exerted bactericidal activity against the two non-susceptible isolates.

Resistance studies. In the 10-day HFIM, a C/T-resistant subpopulation emerged in the susceptible isolate after exposure to the C/T Css of 20 mg/L. Resistance emerged on day 6 at concentrations of 2-, 4-, and eightfold the MIC, and resulted in 1 CFU/mL in 1.6×10^9, 7.8×10^{10}, and 3.9×10^{10}, respectively. The C/T MIC was ≥ 256 mg/L. Compared to the original population, the resistant subpopulation had a lower meropenem MIC (8 vs 16 mg/L) and a lower imipenem MIC (2 vs 8 mg/L). The analysis of mutations within a set of genes involved in antibiotic resistance compared with those already present in the parental isolate (sequencing of bla_{AmpC} gene) revealed a 19-amino acid deletion (K232-G250) in the Ω-loop of AmpC, which has been associated with C/T resistance[25]. No resistant subpopulations were detected following exposure to C/T in CI at a Css of 45 mg/L (Fig. 3).

Drug concentrations. The relationship between observed and predicted C/T concentrations is shown in the Supplemental Material. We assessed the agreement between observed results and predicted results with the Bland–Altman plot. For Css 45 and 80 mg/L difference values have less than a 1.96 of standard deviation (SD) from the mean. On the other hand, for Css 20 mg/L one of the 15 values deviates slightly from 1.96 SD.

Discussion

Optimization of antibiotic treatments based on PK/PD properties is essential in MDR/XDR *P. aeruginosa* infections. C/T has emerged as a promising option in this setting[18]. The standard C/T dose in intermittent infusion regimens can be optimized in high-inoculum infections, but CI may be a better option for achieving PK/PD targets. Our HFIM study compared different Css of C/T in CI against three XDR *P. aeruginosa* ST175 isolates with C/T MICs ranging from 2 to 16 mg/L. The criteria for selecting C/T dosages were to compare lower and higher Css from different doses of C/T that are recommended for difficult-to-treat infections. The ST175 clone was selected because it is the most prevalent in our environment and it has been associated with MDR/XDR isolates involved in nosocomial infections[8].

Few studies have evaluated C/T infusion dosing in the clinical practice. Pilmis et al.[26] compared intermittent infusion and CI in patients with MDR *P. aeruginosa* infections, and concluded that the current recommended dosing regimen provided unsatisfactory coverage. Another Monte Carlo simulations found that extending the duration of C/T infusion improved the probability of target attainment in the treatment of infections caused by MDR *P. aeruginosa* strains with different C/T MICs in patients with different renal functions levels[15]. These results are in consonance with our previous experiments in which different type of infusion (1 h, 4 h and CI) were examined against the same three *P. aeruginosa* isolates. In summary, these studies showed that the CI regimen achieved the highest bacterial reduction even against non-susceptible isolates (an overall reduction of − 4.95 $\log_{10}$ CFU/ml for the CI infusion versus a reduction of − 1.87 and − 2.78 for the 1 h and 4 h infusion, respectively)[27]. Sime et al.[11] described the population PKs of unbound C/T and evaluated the adequacy of recommended dosing regimens in critically ill patients without renal impairment. They concluded that a loading dose of 1 g/0.5 g followed by 3 g/1.5 g in CI was adequate for empirical coverage of a T > MIC target of 100%. In our study, however, CI of 3 g of ceftolozane resulted in the emergence of C/T resistance, indicating that dosing according to PK/PD parameters is important for improving clinical management.

The administration of C/T at a Css of 45 mg/L achieved a reduction in bacterial density in both susceptible and resistant isolates. It also exerted a bactericidal effect regardless of the C/T MICs of the isolates. This sustained suppression of bacterial growth suggests that C/T in CI may achieve concentrations above the susceptibility breakpoint for a longer period of time. This would be particularly important for *P.* aeruginosa isolates with higher C/T MIC values.

Optimization of Css in a CI regimen is necessary to achieve an optimal therapeutic effect. A retrospective study analyzing the performance of C/T in patients with XDR *P. aeruginosa* infections, most of whom were receiving CI, found that 66% achieved supratherapeutic levels[28]. These results highlight the importance of monitoring C/T plasma concentrations when aiming to optimize treatment[28]. We performed a 10-day HFIM study to determine whether a low CI C/T dosing regimen would be as effective as the standard regimen against the C/T-susceptible isolate ST175 (10-023) or possibly contribute to the selection of C/T-resistant subpopulations. We found that a Css of 20 mg/L clearly failed to prevent the emergence of resistance, whereas a Css of 45 mg/L had a bactericidal effect. Sequencing of bla_{AmpC} gene in the resistant subpopulation that emerged revealed a

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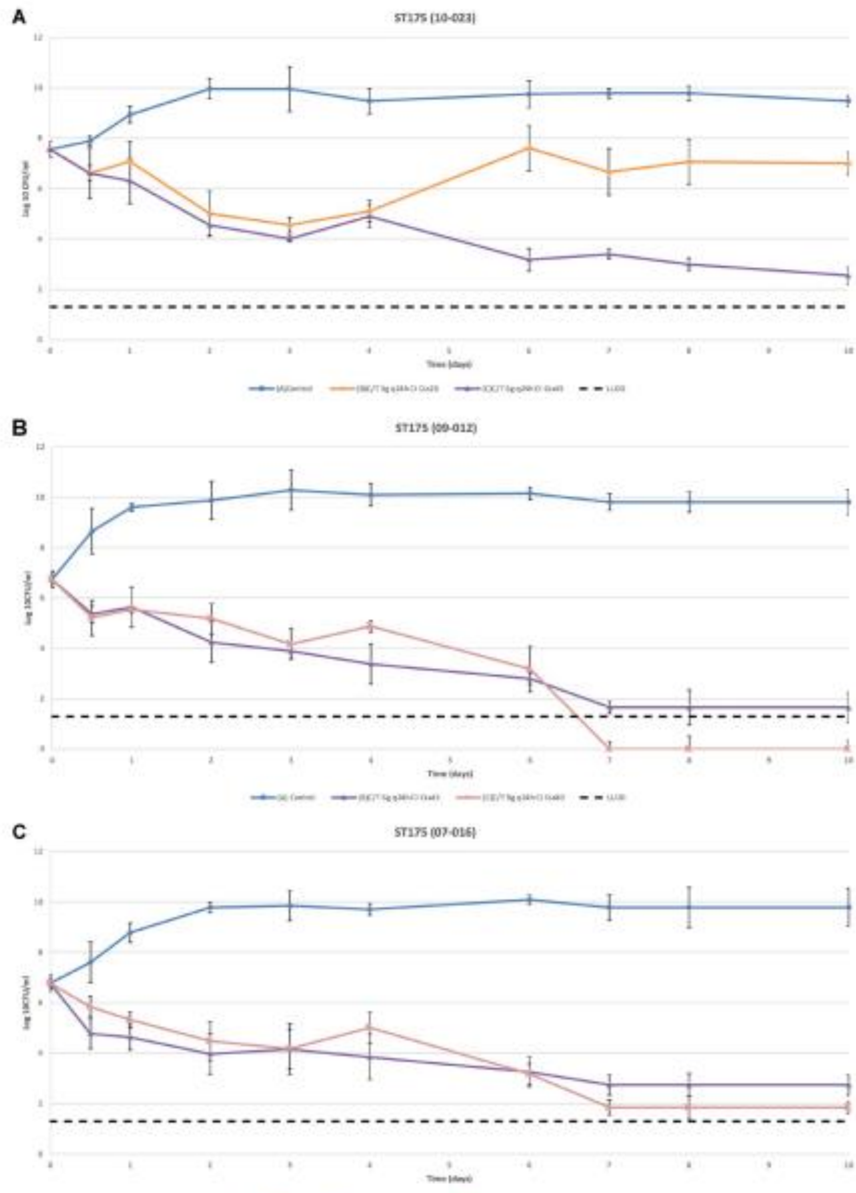

Figure 2. Mean reduction in bacterial density during the 10-day HFIM assays with ST175 (10-023), ST175 (09-012) and ST175 (07-016) isolates treated with different Css of C/T (20, 45 and 80 mg/L) in CI. Respective C/T MIC values of 2, 8 and 16 mg/L. C/T, ceftolozane/tazobactam; CI, continuous infusion; Css, steady-state concentration; LLOD, lower limit of detection; MIC, minimum inhibitory concentration.

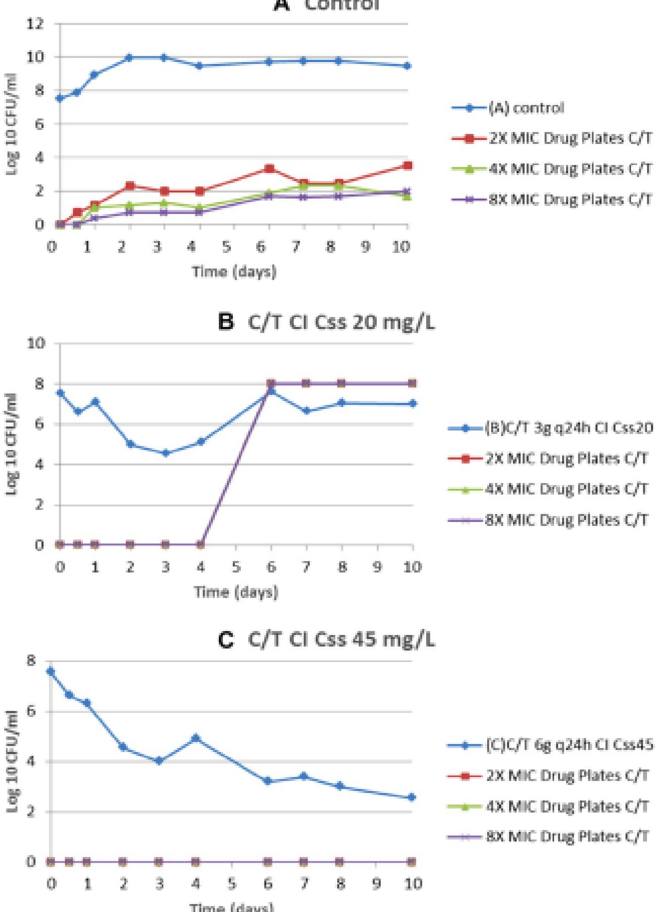

Figure 3. Emergence of C/T resistance in the ST175 (10-023) isolate using Css of 20 and 45 mg/L in CI. C/T, ceftolozane/tazobactam; CI, continuous infusion; Css, steady-state concentration; MIC, minimum inhibitory concentration.

19-amino acid deletion (K232-G250) in the Ω-loop of AmpC, supporting previous reports of a link to C/T resistance[25]. The C/T-resistant subpopulation was also associated with a decrease in meropenem and imipenem MIC values. This phenomenon of partial reversal of carbapenem resistance concomitant with the acquisition of C/T resistance has been previously reported[25]. Bacterial antibiotic susceptibility is therefore dynamic and may be influenced by a gain of resistance in other antibiotics[29].

Infections caused by *P. aeruginosa* isolates with C/T MIC values above 2 mg/L have been associated with poor outcomes when treated with a standard C/T dosing regimen[30]. The use of higher doses (up to 6 g/3 g every 24 h), mainly against non-susceptible strains, has not been found to produce adverse effects[11]. In this context, we evaluated a CI C/T regimen with a Css of 80 mg/L as an option for optimizing the treatment of infections caused by resistant *P. aeruginosa* strains. Our results showed that this higher dose displays a slight advantage than the currently recommended regimen, particularly in the case of the isolate with the intermediate C/T MIC, in which the eradication of the bacterial population was achieved.

This study had some limitations. First, we only studied three *P. aeruginosa* isolates, although they are representative of the different ranges of C/T susceptibility in our environment. Second, despite the use of clinical parameters, we were unable to examine toxicity and infection site effects in vitro, or to determine the contribution of the immune system to bacterial killing, as host immunity could, to a certain extent, modify PD targets. Nonetheless, this absence of immunity means our findings can be extrapolated to immunocompromised patients.

Finally, it should be clarified that exposure to tazobactam was not considered although it has a limited role in ceftolozane's activity against *P. aeruginosa*.

In summary, our results show that C/T in CI at Css of 45 mg/L leads to a decrease in bacterial burden in *P. aeruginosa* and is useful against non-susceptible isolates (with a MIC of 8 and 16 mg/L). CI at a Css of 80 mg/L had the strongest bactericidal effect. Administration of the suboptimal Css of 20 mg/L resulted in the emergence of C/T resistance in the susceptible isolate (MIC 2 mg/L). Antimicrobial regimens can be individually optimized by adjusting antibiotic doses to both C/T MIC values and PK/PD targets. Therapeutic drug monitoring would favour better clinical management and help prevent the emergence of antibiotic resistance.

Received: 3 August 2021; Accepted: 3 November 2021
Published online: 12 November 2021

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Acknowledgements
We thank The Institute for Clinical Pharmacodynamics (ICPD), Schenectady, NY, and the Infectious Pathology and Antimicrobials Research Group (IPAR), Institute Hospital del Mar d'Investigacions Mèdiques (IMIM), for their support.

Author contributions
Conceptualization: M.M.M.; Methodology: M.M.M., S.D., N.P., L.F. A.; Formal analysis and investigation: M.M.M., S.D.; Writing—original draft preparation: M.M.M., S.D., L.F.A., A.A.-B.; Writing—review and editing: all authors; Funding acquisition: M.M.M., J.P.H.; Supervision: all authors.

Funding
This study was partially supported by the Ministerio de Economía y Competitividad of Spain, Instituto de Salud Carlos III. FEDER PI16/00669, PI17/00251, PI18/0076 and the Spanish Network for Research in Infectious Diseases (REIPI RD16/0016).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-01784-4.

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4.5. Comparison of ceftolozane-tazobactam infusion regimens in a hollow-fiber infection model against extensively drug-resistant *Pseudomonas aeruginosa* isolates.

Microbiology Spectrum 2022; 10(3): e0089222.
DOI: 10.1128/spectrum.00892-22.

RESEARCH ARTICLE

Comparison of Ceftolozane/Tazobactam Infusion Regimens in a Hollow-Fiber Infection Model against Extensively Drug-Resistant *Pseudomonas aeruginosa* Isolates

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ABSTRACT The aim of this study was to compare the efficacy of intermittent (1-h), extended (4-h), and continuous ceftolozane-tazobactam (C/T) infusion against three extensively drug-resistant (XDR) sequence type (ST) 175 *P. aeruginosa* isolates with different susceptibilities to C/T (MIC = 2 to 16 mg/L) in a 7-day hollow-fiber infection model (HFIM). C/T in continuous infusion achieved the largest reduction in total number of bacterial colonies in the overall treatment arms for both C/T-susceptible and -resistant isolates. It was also the only regimen with bactericidal activity against all three isolates. These data suggest that continuous C/T infusion should be considered a potential treatment for infections caused by XDR *P. aeruginosa* isolates, including nonsusceptible ones. Proper use of C/T dosing regimens may lead to better clinical management of XDR *P. aeruginosa* infections.

IMPORTANCE Ceftolozane-tazobactam (C/T) is an antipseudomonal antibiotic with a high clinical impact in treating infection caused by extensively drug-resistant (XDR) *Pseudomonas aeruginosa* isolates, but resistance is emerging. Given its time-dependent behavior, C/T continuous infusion can improve exposure and therefore the pharmacokinetic/pharmacodynamic target attainment. We compared the efficacy of intermittent, extended, and continuous C/T infusion against three XDR ST175 *P. aeruginosa* isolates with different C/T MICs by means of an *in vitro* dynamic hollow-fiber model. We demonstrated that C/T in continuous infusion achieved the largest reduction in bacterial density in the overall treatment arms for both susceptible and resistant isolates. It was also the only regimen with bactericidal activity against all three isolates. Through this study, we want to demonstrate that developing individually tailored antimicrobial treatments is becoming essential. Our results support the role of C/T level monitoring and of dose adjustments for better clinical management and outcomes.

KEYWORDS ceftolozane/tazobactam, hollow-fiber, PK/PD, XDR, *Pseudomonas aeruginosa*

Antibiotic resistance has led to increased morbidity and mortality worldwide, limiting treatment options and contributing to the emergence and selection of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria (1–3). MDR/XDR *Pseudomonas*

Editor Aude A. Ferran, INTHERES
Ad Hoc Peer Reviewer Gloria Wong, University of Queensland
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The authors declare no conflict of interest.
Received 9 March 2022
Accepted 20 May 2022

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aeruginosa isolates are particularly concerning as they are the leading cause of nosocomial infections and are independently associated with in-hospital mortality (4). *P. aeruginosa* has intrinsic resistance to a broad range of antibiotics. This poses a major risk for resistance development due to mutations in chromosomal genes or horizontal gene transfer (1). This is a concern due to the risk of dissemination. Antibiotic resistance is very common in *P. aeruginosa* because of high spontaneous mutation rates, especially in infections with a high bacterial load. *P. aeruginosa* infections pose a medical challenge, particularly when caused by high-risk clones, which are present in hospitals around the world and are directly linked to difficult-to-treat infections (5–7). One example is the ST175 clone, which is particularly common in a number of European countries (8). This clone is also the most common XDR isolate in Spain; the main resistance mechanisms described for ST175 are AmpC hyperproduction and OprD deficiency due to mutations (8). The limited number of treatment options for infections caused by high-risk clones increases the risk of inadequate clinical management. The short-term outlook is not very encouraging due to the lack of a development pipeline for antipseudomonal agents. That said, progress has been made in the development of new molecules in the past year, and new combinations of antibiotics and beta-lactamase inhibitors have appeared.

Ceftolozane-tazobactam (C/T) has emerged as a promising option for treating infections caused by MDR/XDR *P. aeruginosa* isolates resistant to all first-line agents (ticarcillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, and ciprofloxacin) (9). C/T combines ceftolozane, a novel cephalosporin, with the beta-lactamase inhibitor tazobactam (7). The licensed dosing regimen is a 1-h infusion of 1.5 g every 8 h for complicated urinary tract infections and intra-abdominal infections and 3 g every 8 h for hospital-acquired bacterial pneumonia, including ventilator-associated bacterial pneumonia (10).

Because C/T has time-dependent pharmacokinetic (PK) properties, the most suitable pharmacodynamic (PD) parameter for predicting its bacteriological efficacy is $f\%T > MIC$, which is the percentage of the dosing interval (%T) in which plasma free drug concentrations remain above the MIC. In C/T, this is approximately 40 to 50% (7, 11). The currently recommended dosing regimen might be inadequate to treat infections caused by MDR/XDR *P. aeruginosa* isolates with a C/T MIC above the susceptibility breakpoint of 4 mg/L. In such cases, combination therapy or alternative dosing regimens may need to be individualized to optimize treatment (12).

PK/PD studies are needed to define optimal treatments for XDR *P. aeruginosa* infections. *In vitro* methods can be used to examine interactions between drugs and bacteria to optimize antibiotic use. The hollow-fiber infection model (HFIM) is a dynamic two-compartment method that makes it possible to conduct experiments mimicking human PK under biosafety conditions. It can complement or substitute animal models of infection while overcoming the limitations of static models. In HFIM experiments, bacteria are exposed over time to clinically relevant, fluctuating drug concentrations achieved by repeated dosing and constant elimination.

The aim of this study was to evaluate the efficacy of three C/T dosing regimens against three XDR *P. aeruginosa* isolates in an *in vitro* HFIM. We compared standard intermittent infusion over 1 h, extended infusion over 4 h, and continuous infusion. The isolates were from the ST175 clone and had different C/T MICs (2, 8, and 16 mg/mL).

RESULTS

***In vitro* susceptibility and resistance mechanisms.** The *P. aeruginosa* (10-023) isolate was susceptible to C/T (MIC, 2 mg/L) and resistant to the other β-lactams due to OprD inactivation and AmpC hyperproduction (13). The *P. aeruginosa* (09-012) isolate was intermediate to C/T (MIC, 8 mg/L), attributable to OprD inactivation, AmpC hyperproduction, and a mutation in PBP3 (R504C) that has been linked to increased β-lactam resistance (13). The *P. aeruginosa* (07-016) isolate was resistant to C/T (MIC, 16 mg/L); in this case, resistance was attributed to production of a class A carbapenemase GES-5 coupled with OprD inactivation (13).

Estimated frequency of mutants. The mean density of the *P. aeruginosa* (10-023) (C/T MIC of 2 mg/L) drug-resistant population exposed to C/T at 2, 4, and 8 times the baseline MIC (corresponding to 4, 8, and 16 mg/L) was 1 CFU in 3.3×10^8, 4.6×10^8, and 1.6×10^9

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CFU/mL, respectively. The mean density of the *P. aeruginosa* (09-012) (C/T MIC of 8 mg/L) drug-resistant population exposed to C/T at 2, 4, and 8 times the baseline MIC (corresponding to 16, 32, and 64 mg/L) was 1 CFU in 7.2×10^7, 1.09×10^8, and 1.15×10^8 CFU/mL, respectively. Finally, the mean density of the *P. aeruginosa* (07-016) (C/T MIC of 16 mg/L) drug-resistant population exposed to C/T at 2, 4, and 8 times the baseline MIC (corresponding to 32, 64, and 128 mg/L) was 1 CFU in 3.5×10^7 and 3.75×10^7 CFU/mL. Mutant frequency could not be determined at concentrations 8 times the baseline MIC due to a lack of growth on the drug-containing plates.

HFIM. The mean numbers of bacterial colonies grown for the three C/T regimens over the 7-day HFIM study are shown in Fig. 1. The three isolates, with an initial mean inoculum of 7.26 $\log_{10}$ CFU/mL, were analyzed. Table 1 shows the mean total reduction in the number of colonies (log difference at 24 h) for each regimen compared with that of the control.

The total number of bacterial colonies grown for *P. aeruginosa* (10-023) on day 7 was 4.74 ± 0.19 $\log_{10}$ CFU/mL for intermittent infusion, 3.53 ± 0.1 $\log_{10}$ CFU/mL for extended infusion, and 2.54 ± 0.05 $\log_{10}$ CFU/mL for continuous infusion. The respective reductions were 2.26 ± 0.19, 3.47 ± 0.10, and 4.46 ± 0.05 $\log_{10}$ CFU/mL (Fig. 1A).

The total number of bacterial colonies grown for *P. aeruginosa* (09-012) on day 7 was 4.57 ± 0.04, 3.91 ± 0.37, and 1.65 ± 0.18 $\log_{10}$ CFU/mL for intermittent, extended, and continuous dosing, respectively (Fig. 1B). An overall reduction in number of colonies was observed up to day 6; regrowth was detected in the populations treated with intermittent infusion and extended infusion. The continuous C/T infusion regimen, by contrast, was associated with a continuous reduction. The reductions achieved over the 7 days were 2.53 ± 0.04 $\log_{10}$ CFU/mL for intermittent infusion, 3.19 ± 0.37 $\log_{10}$ CFU/mL for extended infusion, and 5.45 ± 0.18 $\log_{10}$ CFU/mL for continuous infusion.

Finally, the total number of bacterial colonies grown for *P. aeruginosa* (07-016) on day 7 was 6.85 ± 0.22 $\log_{10}$ CFU/mL for intermittent infusion, 5.98 ± 0.33 for extended infusion, and 2.74 ± 0.37 $\log_{10}$ CFU/mL for continuous infusion (Fig. 1C). This C/T-resistant isolate displayed a mean reduction of 4.79 $\log_{10}$ CFU/mL at 8 h for all regimens. There was a regrowth during the first 3 days for the intermittent and extended regimens, and after this point, colony numbers remained relatively stable for the intermittent and extended regimens, with a respective overall reduction of 0.83 ± 0.22 and 1.7 ± 0.33 $\log_{10}$ CFU/mL. The corresponding reduction achieved with continuous infusion was 4.94 ± 0.37 $\log_{10}$ CFU/mL.

Extended infusion had a bactericidal effect on *P. aeruginosa* (10-023) and *P. aeruginosa* (09-012), while intermittent infusion showed no bactericidal activity.

The reductions achieved in overall bacterial density using the different regimens are compared and shown as the log difference on day 7 and the log ratio (LR) of area under the curve for CFU (AUCFU) in Table 1. Relative to control (reference), all regimens achieved greater than 2-log reduction against the three isolates (2.90 to 3.69), and the higher AUCFU reductions were accomplished with the continuous infusion (CI) regimens in the three isolates studied. The regimen of C/T in continuous infusion compared with the regimen of C/T in intermittent infusion achieved greater than 1-log reduction against the three isolates. Compared with the regimen of C/T in extended infusion, the reductions accomplished with C/T in continuous infusion were greater than 1-log against *P. aeruginosa* (09-012) and *P. aeruginosa* (07-016). Referenced to the reduction achieved with C/T in extended infusion versus intermittent infusion, the reductions were less than 1-log reduction (0.10 to 0.87), against the three isolates. Regarding the $f\%T > MIC$ parameter, in the overall regimens of the HFIM performed, the $f\%T > MIC$ accomplished was greater than 98%.

Drug concentrations. The relationship between observed and predicted C/T concentrations over the 7 days is shown in Table 2. The simulated drug exposures were satisfactory, with R^2 values of 0.920 for intermittent infusion, 0.921 for extended infusion, and 0.905 for continuous infusion (Fig. 2). For each point of predicted C/T concentration, the observed concentration increased by between 1.03 and 1.05 (mg/L).

DISCUSSION

Optimization of antimicrobial PK/PD properties when treating XDR *P. aeruginosa* infections is crucial in our setting. The appearance of novel antibiotic products such as

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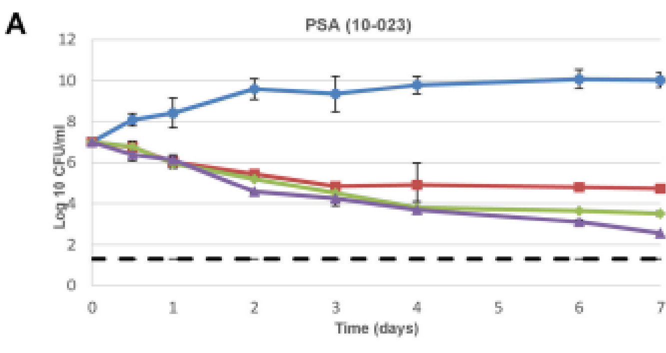

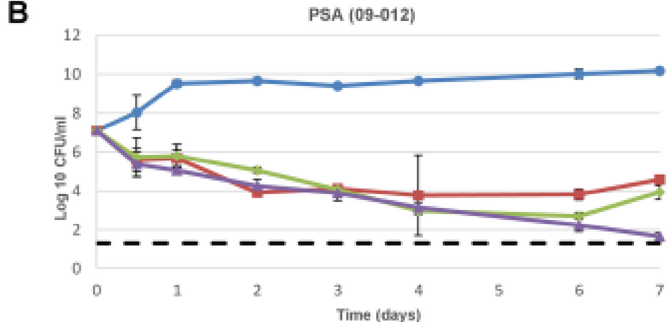

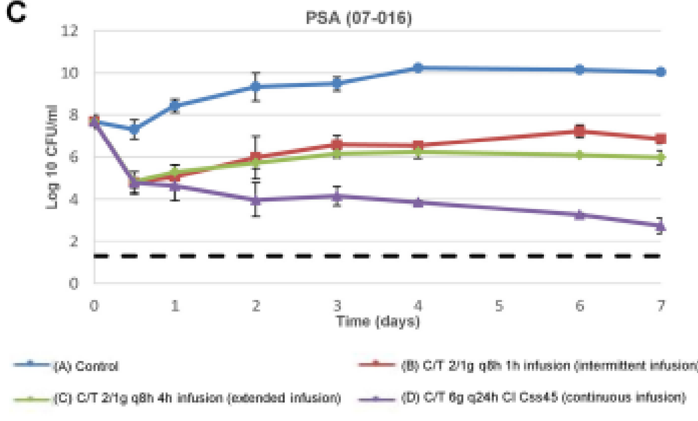

FIG 1 Mean reduction in number of bacterial colonies (CFU/mL) over 7 days in an *in vitro* HFIM model testing three C/T infusion regimens (1-h, 4-h, and continuous infusion) against three XDR ST175 *P. aeruginosa* isolates: (A) *P. aeruginosa* (10-023), (B) *P. aeruginosa* (09-012), and (C) *P. aeruginosa* (07-016), with respective C/T MIC values of 2, 8, and 16 mg/L. Experiments were conducted in duplicate. C/T, ceftolozane/tazobactam; CI, continuous infusion; Css, steady-state concentration; q8h, every 8 h; LLOD, lower limit of detection.

C/T paves the way toward a treatment era in which individual characteristics will be taken into account to achieve optimized strategies. C/T is a promising option for the treatment of *P. aeruginosa* infections (14). Because it is a time-dependent antimicrobial, extended infusion could improve the probability of optimal PK/PD target attainment.

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TABLE 1 Mean overall reduction in number of bacterial colonies grown with alternative C/T infusion regimens for each ST175 isolate; parameters included $\log_{10}$ CFU/mL ± standard deviation, and LR of AUCFU

	P. aeruginosa (10-023)		*P. aeruginosa* (09-012)		*P. aeruginosa* (07-016)	
Infusion regimen	Log diff day 7[a]	LR of AUCFU[b]	Log diff day 7	LR of AUCFU	Log diff day 7	LR of AUCFU
C/T 2/1 g q8h 1-h infusion vs control	−2.26 ± 0.19	−3.37	−2.53 ± 0.04	−3.66	−0.83 ± 0.22	−2.90
C/T 2/1 g q8h 4-h infusion vs control	−3.47 ± 0.10	−3.38	−3.19 ± 0.37	−3.64	−1.7 ± 0.33	−3.15
C/T 6 g q24h CI Css45 vs control	−4.46 ± 0.05	−3.53	−5.45 ± 0.18	3.69	−4.94 ± 0.37	−3.24
C/T 6 g q24h CI Css45 vs C/T 2/1 g q8h 1-h infusion	−2.2 ± 0.1	−1.01	−2.92 ± 0.01	−1.52	−4.11 ± 0.12	−2.1
C/T 6 g q24h CI Css45 vs C/T 2/1 g q8h 4-h infusion	−0.99 ± 0.33	−0.65	−2.26 ± 0.2	−1.23	−3.24 ± 0.05	−1.85
C/T 2/1 g q8h 4-h infusion vs C/T 2/1 g q8h 1-h infusion	−1.21 ± 0.05	−0.87	−0.66 ± 0.15	−0.10	−0.87 ± 0.28	−0.15

[a]Log difference at the end of the assay for each regimen compared with the control.
[b]The log difference is presented as the log ratio (LR), which is used to compare any number of $\log_{10}$ CFU of two regimens (test/reference). AUCFU, area under the curve for CFU; C/T, ceftolozane-tazobactam; CI, continuous infusion; Css, steady-state concentration; q8h, every 8 h.

We used the HFIM system to compare three C/T infusion regimens against three XDR *P. aeruginosa* ST175 isolates with C/T MIC values ranging from 2 to 16 mg/L. The ST175 clone has been associated with MDR/XDR isolates; it is a common hospital contaminant and causes difficult-to-treat respiratory tract infections in patients with cystic fibrosis or chronic obstructive pulmonary disease (14, 15).

Our *in vitro* study showed that overall, C/T in continuous infusion reduced the density of susceptible and resistant *P. aeruginosa* isolates, reinforcing the idea that this mode of administration results in concentrations that remain above the susceptibility breakpoint for longer. The reduction in density was even more evident in less-susceptible isolates with higher MIC values, where continuous infusion of C/T led to sustained suppression of the bacterial population, outperforming both the intermittent and extended dosing regimens. In addition, it was the only regimen with bactericidal activity against all three isolates, supporting its potential superiority and suggesting that the currently recommended regimen does not provide adequate coverage against MDR/XDR *P. aeruginosa* isolates. Our results are consistent with those reported by Pilmis et al. (16), who found that compared with intermittent administration, C/T in continuous infusion was associated with a higher probability of target attainment (>90%) for MDR *P. aeruginosa* isolates with a C/T MIC of 4 mg/L. Sime et al. (10) showed that a C/T dosing regimen of 3 g every 8 h was associated with relatively low fractional target attainment in patients with severe augmented renal clearance, which could be problematic when treating *P. aeruginosa* infections caused by MDR isolates that are potentially less susceptible to this antibiotic combination (17, 18).

Findings from other PK/PD simulation studies suggest that optimal β-lactam exposure is rapidly obtained via continuous or extended infusion (19, 20). Natesan et al. (12), using Monte Carlo simulation to determine which C/T dosing regimens were most likely to optimize probability of target attainment for MDR *P. aeruginosa* isolates with different C/T MICs, found that extended infusion was superior in certain scenarios. In our study, the extended dosing regimen showed only a slight advantage over the currently recommended 1-h regimen (final mean number of bacterial colonies of 5.39 versus 4.48 CFU/mL).

If it is confirmed that continuous infusion of C/T achieves the greatest reduction in MDR/XRD *P. aeruginosa* populations, optimization of steady-state concentrations will be necessary to achieve optimal clinical outcomes and prevent the selection of C/T-resistant subpopulations (21, 22). In a previous study by our group, a steady-state concentration of 45 mg/L reduced bacterial density and prevented the emergence of C/T resistance in HFIM assays

TABLE 2 Observed versus predicted antibiotic concentrations achieved in each HFIM model[a]

	Free peak concn (mg/L) ± SD		Free trough concn (mg/L)/Css ± SD	
Dosing regimen	Predicted value	Observed value	Predicted value	Observed value
C/T 2/1 g q8h 1-h infusion	74.45	61.96 ± 6.80	14.77	25.67 ± 3.7
C/T 2/1 g q8h 4-h infusion	54.55	53.10 ± 7.92	19.7	27.29 ± 5.63
C/T 6 g q24h CI			45	47.29 ± 5.43

[a]Data are presented as the mean concentration ± standard deviation. Css, steady-state concentration; q8h 1-h, infusion over 1 h every 8 h (intermittent infusion); q8h 4-h, infusion over 4 h every 8 h (extended infusion); SD, standard deviation.

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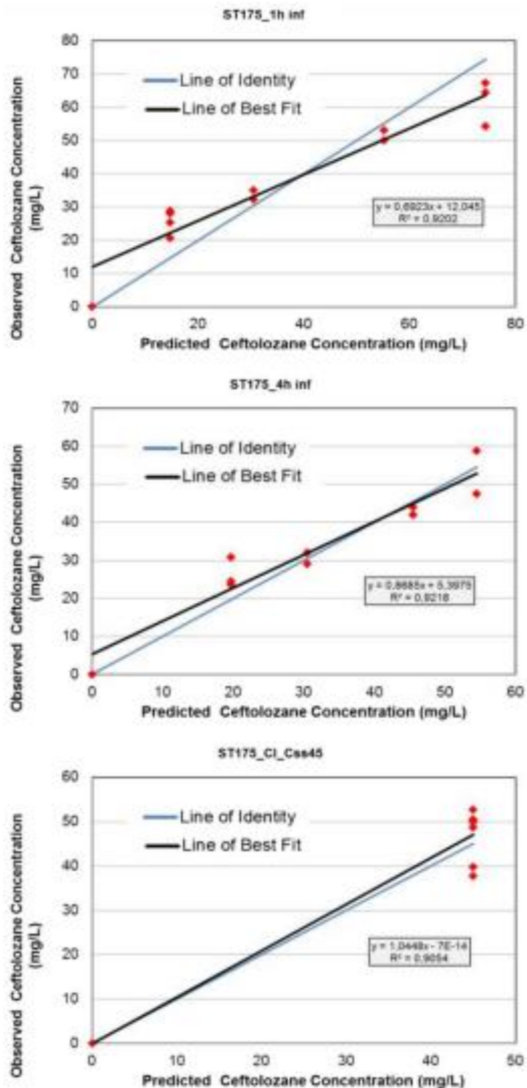

FIG 2 Relationship between observed and predicted ceftolozane concentrations for intermittent (1-h), extended (4-h), and continuous C/T infusion regimens in the overall experiments. inf, infusion; CI, continuous infusion; Css, steady-state concentration.

with XDR *P. aeruginosa* isolates (22). Escolà-Vergé et al. (23) reported the development of resistance with the use of low-dose (1.5 g every 8 h) and high-dose (3 g every 8 h) C/T in the treatment of MDR *P. aeruginosa* infections. The increase in MIC values ranged from 8-fold to >85-fold.

Although the study parameters (dose, dosing interval, and duration of experiment) were designed to simulate clinical exposure, our study has a number of limitations that should be taken into account. Because it was an *in vitro* study, we were unable to examine toxicity, immune responses, or injection-site PK/PD effects. Moreover, some PK parameters,

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such as half-life and clearance, could be altered in some critically ill patients. We also studied just three isolates, although they are representative of C/T susceptibility ranges in our environment.

In summary, our findings show that C/T in continuous infusion achieves a greater overall reduction in bacterial burden than intermittent or extended dosing regimens, particularly in the case of nonsusceptible XDR *P. aeruginosa* isolates. The current recommended dosing regimen would appear to offer inadequate coverage for optimal PK/PD target attainment. Continuous infusion regimens are potentially useful and should be investigated further. The findings of this *in vitro* study suggest that correct use of C/T dosing regimens could lead to better clinical management of *P. aeruginosa* infections caused by XDR isolates.

MATERIALS AND METHODS

Bacterial isolates and resistance mechanisms. We selected three XDR ST175 *P. aeruginosa* clinical isolates representative of the clones and resistance mechanisms in our environment: *P. aeruginosa* (10-023), *P. aeruginosa* (09-012), and *P. aeruginosa* (07-016). The isolates were collected from a collection of 150 XDR clinical isolates from nine hospitals located in six different Spanish regions in the context of a multicenter clinical study (EudraCT 2013-005583-25, PI JP Horcajada). They were obtained from different infection sources and stored at −80°C (storage vials with 10% glycerol). Fresh isolates were subcultured twice on 5% blood agar plates for 24 h at 35°C before each experiment. The isolates had been previously characterized for molecular epidemiology purposes using pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and whole-genome sequencing (13). Expression levels of *ampC*, *oprD*, *mexB*, *mexD*, *mexF*, and/or *mexY* were determined using RT-PCR (reverse transcription PCR); expression of outer membrane proteins (including OprD) was determined by SDS-PAGE, while penicillin-binding protein (PBP) profiles were determined in a competition assay with fluorescent penicillin (Bocillin Fl). The main target mutations were sequenced using previously described primers. Clonal relatedness was evaluated by PFGE. Finally, whole-genome sequencing was performed in the three isolates (13).

Antibiotics. C/T (Zerbaxa; Merck & Co, Inc., Kenilworth, NJ; lot number SO15404; expiration date, August 2020) was provided by Merck & Co., Inc. (Kenilworth, NJ). The antibiotic solutions were prepared according to CLSI guidelines (24). The dosing regimens were within a dose range based on previously determined maximum concentration of drug in serum (C_{max}) and area under the curve (AUC) (21). Three regimens were simulated: 2 g/1 g every 8 h over 1 h (intermittent infusion) to reach a free C_{max} (fC_{max}) of 75 mg/L, 2 g/1 g every 8 h over 4 h (extended infusion) to reach an fC_{max} of 55 mg/L (10), and continuous infusion to reach a steady-state concentration of 45 mg/L. The simulated elimination half-life for ceftolozane was 3 h (14, 21). Although tazobactam is present in the pharmaceutical formulation, exposure to this drug was not considered since it has a limited role in ceftolozane's activity against *P. aeruginosa* (25). C/T concentrations were validated by high-performance liquid chromatography (HPLC) (26).

Estimated frequency of spontaneous mutants. Preexisting mutations in genes associated with C/T resistance may increase the risk of C/T-resistant subpopulations developing in the total bacterial population. The frequency of spontaneous mutants conferring C/T resistance was estimated for all isolates by plating 4 mL of log-phase growth suspension onto agar containing ceftolozane at concentrations of 2, 4, and 8 times the baseline MIC and tazobactam at a fixed concentration of 4 mg/L. The experiments were performed in duplicate. After 48 h of incubation, the bacterial concentration within each suspension was determined by quantitative culture. The ratio of growth on the drug-containing plates to that of the starting inoculum provided an estimate of the frequency of mutants conferring drug resistance within each population.

***In vitro* antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed according to the CLSI guidelines (24) for broth microdilution using cation-adjusted Mueller-Hinton broth (CAMHB). The ceftolozane susceptibility test was conducted alone and in combination with a fixed tazobactam concentration (4 mg/L).

HFIM. The efficacy of the three C/T infusion regimens against *P. aeruginosa* (10-023), *P. aeruginosa* (09-012), and *P. aeruginosa* (07-016) was investigated in a 7-day HFIM study as described previously (14, 27). In the HFIM, bacteria are exposed over time to clinically relevant, fluctuating drug concentrations achieved by repeated dosing and constant elimination. Four arms were analyzed: no treatment (control), intermittent infusion, extended infusion, and continuous infusion. Polyethersulfone hemofilters were used as the hollow-fiber cartridges with a volume of 50 mL (Aquamax HF03, Nikkiso, Belgium). Experiments were conducted in duplicate at 37°C in a humidified incubator set. Separate infusion pumps were used to pump each of the C/T regimens into the central reservoir to reach predicted concentrations simulating free drug PK profiles in humans. Fresh drug-free growth medium CAMHB was continuously infused into the central reservoir to dilute and simulate drug elimination in humans. An equal volume of drug-containing medium was concurrently removed from the central reservoir to maintain an isovolumetric system. An overnight culture of each isolate was diluted with CAMHB and further incubated at 37°C in a water bath shaker to reach early log-phase growth. The density of the growth broth was calculated for an initial inoculum of 10^7 to 10^8 CFU/mL using a spectrophotometer at 630 nm. The extracapillary space of each HFIM was inoculated with 50 mL of the bacterial suspension to simulate high-inoculum infections. The bacteria were confined to the extracapillary space but exposed to fluctuating drug concentrations from the HFIM cartridge through an internal circulatory pump in the bioreactor loop. At 0, 8, 24, 48, 72, 96, 144, and 168 h, bacterial samples were collected from the cartridges, washed, centrifuged twice at 13,000 rpm for 3 min, and then reconstituted with sterile saline solution to the same original volume

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to minimize drug carryover. Serially diluted samples were quantitatively cultured onto drug-free Trypticase soy agar (BBL TSA II, Becton, Dickinson) plates to determine the count of the total bacterial population ($\log_{10}$ CFU/mL). The inoculated plates were incubated in a humidified incubator (37°C) for 24 h, the bacterial colonies were visually counted, and the number of bacterial cells in the original sample was calculated based on the dilution factor. The lower limit of detection (LLOD) was 1.3 $\log_{10}$ CFU/mL. Bactericidal activity was defined as a reduction of 3 $\log_{10}$ CFU/mL from the initial bacterial density (28).

Drug concentrations. Antibiotic samples were collected from the peripheral compartment of the HFIM system at different time points over the first 48 h and once a day until the end of the study. Samples were stored at −80°C until analysis. Samples were taken to validate ceftolozane concentrations. All exposures simulating free drug PK in humans were based on the half-life of ceftolozane. Samples were taken to report free peak and trough antibiotic values, and concentrations were analyzed by HPLC (26).

Descriptive statistics. The difference in area under the curve for CFU (AUCFU) was calculated as described previously (29). We calculated an end-of-treatment endpoint, the log ratio of AUCFU (LR), as $LR = \log_{10}(AUCFUtest/AUCFUreference)$, where the reference regimen was the growth control. An LR value of −1 indicated a 90% (10-fold) reduction in overall bacterial density, while a value of −2 indicated a 99% (100-fold) reduction (28, 29).

ACKNOWLEDGMENTS

We thank The Institute for Clinical Pharmacodynamics (ICPD), Schenectady, NY and the Infectious Pathology and Antimicrobials Research Group (IPAR), Institute Hospital del Mar d'Investigacions Mèdiques (IMIM) for their support.

We declare no conflict of interest.

Conceptualization: Maria Milagro Montero; methodology: Maria Milagro Montero, Sandra Domene, Núria Prim, Lorena Ferrer Alapont; formal analysis and investigation: Maria Milagro Montero, Sandra Domene; writing—original draft preparation: Maria Milagro Montero, Sandra Domene, Lorena Ferrer Alapont; review and editing: all authors; funding acquisition: Maria Milagro Montero, Juan Pablo Horcajada; supervision: all authors. All authors have read and agreed to the published version of the manuscript.

This study was partially supported by the Ministerio de Economía y Competitividad of Spain, Instituto de Salud Carlos III FEDER PI16/00669, PI17/00251, PI18/0076, and the Spanish Network for Research in Infectious Diseases (REIPI RD16/0016).

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5. RESULTS & DISCUSSION

MDR/XDR *P. aeruginosa* is included in the pathogens for which the situation is considered critical by the World Health Organization (3,4). Currently, there are only a few effective treatments when facing these infections. Consequently, antibiotic treatment optimization is a constant concern.

In the present study we evaluated the effect of different antibiotic regimens against collection of isolates of XDR *P. aeruginosa* belonging to several clones, including XDR high-risk clones. These isolates had been previously collected from nine Spanish hospitals in the multicenter COLIMERO trials and characterized at a molecular level using pulsed field gel electrophoresis, multilocus sequence typing and whole-genome sequencing (43). The selected isolates provide a representative profile of all the clones and resistance mechanisms have been detected in the Spanish trial.

First of all, we evaluated the activity of different antipseudomonal antibiotics, alone or in combination, used in clinical practice by checkerboard and time-kill curves, in order to identify the most effective one. In previous studies polymyxin-carbapenem combinations were proposed against MDR/XDR Gram-negative infections to enhance the therapeutic response and minimize potential polymyxin resistance, especially useful when there were no other therapeutic options (55). Other combinations with reported synergy against MDR *P. aeruginosa* are including colistin plus doripenem (52,55,78), colistin-ceftazidime (79), colistin-rifampicin (80,81), meropenem-levofloxacin (82), and colistin-imipenem (83).

Through checkerboard experiments results, additive and synergistic combinations were selected in order to validate them with time-kill curves against the three most prevalent high-risk clones (ST175, ST111, ST235). The most effective combination found - colistin plus meropenem – was then validated in the entire collection of XDR *P. aeruginosa* isolates. All strains were resistant to meropenem, but three of them were intermediate (MIC 8 mg/L). Just one strain was colistin resistant (MIC 4 mg/L). In time-kill curves, the untreated control failed, for each study regimen when administered alone. The resulting combination with the greatest efficacy,

colistin plus meropenem, showed a synergistic effect against 80% of the 20 strains studied. These results suggest that this therapy could be a potential option in severe infection caused by *P. aeruginosa* high-risk clones.

Due to most of the current XDR *P. aeruginosa* isolates still maintain susceptibility to colistin (84,85), we decided to evaluate combination therapy with C/T and colistin in our collection of 24 representative XDR *P. aeruginosa* isolates. C/T arises as a promising alternative for the treatment for MDR/XDR *P. aeruginosa* because its effectiveness (73). It is less affected by the typical resistance mechanisms associated with this bacterium or emerged mutations such as the PBP-coding genes (63). However, there are many patients with severe infections or with less susceptible strains with higher C/T MIC values, who could benefit from combination therapy. Few studies have examined combination therapy of C/T plus colistin (86,87). Twelve of the selected isolates were resistant to C/T and one of them was resistant to colistin.

The combination was evaluated with time-kill curves, and it has demonstrated superior synergistic or additive effect for C/T plus colistin against 21 of the 24 isolates studied. Furthermore, the combination had bactericidal effect in all cases. Time-kill curves with colistin monotherapy showed a similar pattern in all the isolates, with an initial reduction after 2 h, followed by regrowth in all cases. In contrast, no regrowth was observed for C/T plus colistin combination for any of the isolates, supporting former reports (87).

In a second stage, we studied this combination in three ST175 *P. aeruginosa* isolates by the one-compartment *in vitro* PK/PD model called chemostat (88). ST175 is the most prevalent high-risk clone in our environment and, in this case, had a C/T MIC values of between 2 and 16 mg/L The simulated C/T dosing regimen was 2/1 g every 8 h by intravenous infusion over 1 h (current standard) with a simulated half-life of 3 h (73,88,89). It was assumed that tazobactam would be eliminated at the same half-life of ceftolozane, since it has a limited role in ceftolozane activity against *P. aeruginosa* (90). Colistin was simulated in CI to achieve concentrations of 2 mg/L to mimic plasma colistin concentration-time

profiles in critically ill patients (91). The C/T regimen of 2/1 g every 8 h with a Css of 2 mg/L colistin effectively suppressed the bacterial growth at 24 h. Additive or synergistic interactions were observed for C/T plus colistin against XDR *P. aeruginosa* isolates and particularly against C/T-resistant strains. The combination led to increased activity against XDR *P. aeruginosa* compared with either the agents used as monotherapy.

Besides that, in the resistant studies the emergence of colistin-resistant subpopulation was detected both in the control arm and in the cultures receiving colistin monotherapy. No colistin-resistant subpopulations were detected for the combination. These data are helpful to understand the results, since heteroresistance already could be present, and therefore these isolates killed with the addition of C/T, or resistance could be caused by suboptimal colistin concentrations, and therefore C/T can prevent this resistance development from occurring. Studies investigating resistance development with colistin monotherapy compared with combination therapy have shown suppression or delay of colistin resistance when combination therapy is used (55).
The combination effect observed was probably due to the different mechanisms of action of these two antibiotics (92). Colistin acts against the LPS disrupting the outer membrane causing local disturbance, permeability changes, osmotic imbalance and death cell. The resulting increase in permeability would facilitate uptake of carbapenems inside the cell (93) allowing, for example, C/T or meropenem entrance.

In recent years, the availability of new drugs such as CZA has increased the therapeutic arsenal against Gram-negative pathogens, including XDR *P. aeruginosa* (72). It has activity against ESBL-producers and carbapenem-resistant Enterobacterales, but it is not active against MBL-producers. To preserve the effectiveness of CZA, its clinical use should be avoided in naturally resistant strains and in those carrying MBLs and certain class D beta-carbapenemases (94).

Combination therapy has an important role in these clinical scenarios. We performed a time-kill analysis of the effectiveness of CZA alone and in combination

with other antipseudomonal antibiotics against 21 XDR *P. aeruginosa* isolates. Seven isolates were resistant to CZA, including four MBL-carrying isolates and two class A carbapenemases. CZA showed bactericidal effect in 100% of the CZA-susceptible isolates. Regarding the CZA-resistant isolates, the combination with colistin was additive or synergistic in 100% of the isolates, while the combination with amikacin or aztreonam was additive or synergistic in the 85% of the cases. These findings support that a CZA combination could be useful for treating XDR *P. aeruginosa* infections and highlighted its potential role against CZA-resistant isolates.

Antibiotic resistance contributes to the emergence and selection of XDR *P. aeruginosa* and led to a critical decrease in the availability of alternative antibiotic treatments (1). For this reason, strategies to monitor and prevent the selection of resistance during antibiotic treatment are urgently needed. C/T has emerged as a promising option in this setting, but resistance is emerging (73). C/T combination therapy treatment could be an alternative when the current standard C/T dosing regimen might be insufficient, for example when referring to XDR *P. aeruginosa* with non-susceptible C/T MIC. Another possibility could be C/T alternative dosing regimens that consider the patient's profile. The standard dose for C/T is a 1-h infusion of 1.5 g every 8 h for cUTI and cIAI and 3 g every 8 h for HABP (65). As it is a time-dependent antimicrobial, prolonged infusion may help achieve PK/PD targets. Few studies have evaluated C/T alternatives dosing infusions (65,66,95).

We aimed to compare the efficacy of intermittent (1-h), extended (4-h) and continuous C/T infusion against three XDR ST175 *P. aeruginosa* isolates with different susceptibilities to C/T (MIC between 2 and 16 mg/L) in a hollow-fiber infection model. We had selected ST175 clone because it is the most prevalent in our environment and it has been associated with MDR/XDR isolates being a recognized hospital contaminant (52). Additionally, on a second stage, different Css of C/T in CI against the same ST175 isolates were analyzed.

On the first stage, C/T in CI achieves greater overall reduction in bacterial burden than intermittent or extended dosing regimens, particularly in the case of non-

susceptible XDR *P. aeruginosa* isolates (an overall reduction of -4.95 log_{10} CFU/ml for the CI versus a reduction of -1.87 and -2.78 log_{10} CFU/ml for the 1-h and 4-h infusion, respectively). In addition, it was the only regimen with bactericidal activity against all three isolates. These findings reinforce the idea that this mode of administration works in concentrations that remain above the susceptibility breakpoint for longer.

Once determined that CI of C/T achieves the greatest reduction in XDR *P. aeruginosa* populations, optimization of Css. Will be necessary to achieve optimal clinical outcomes and prevent the selection of C/T-resistant subpopulations. On the second stage, we evaluated different Css of C/T in CI to test the effectiveness of C/T and the emergence of resistance by the hollow-fiber infection model. C/T dosing regimens were simulated to achieve approximate Css of 20, 45 and 80 mg/L (which respectively correspond to 3, 6 and >9 g/ 4.5 every 24 h) (96). Effectiveness was investigated by treating C/T-non-susceptible *P. aeruginosa* isolates with C/T in CI at Css of 45 and 80 mg/L, while resistance was investigated by treating the C/T-susceptible isolate with C/T in CI at Css of 20 and 45 mg/L. Results showed that a Css of 20 mg/L clearly failed to prevent the emergence of resistance, whereas a Css of 45 mg/L had a bactericidal effect. Sequencing of bla_{AmpC} gene in the resistant subpopulation that emerged revealed a deletion in the gene encoding AmpC beta-lactamase, supporting previous reports of a link to C/T resistance (63).

Infections caused by *P. aeruginosa* isolates with C/T MIC values above 2 mg/L have been associated with poor outcomes when treated with a standard C/T dosing regimen (62). The use of higher doses (up to 6/3 g every 24 h), mainly against non-susceptible strains, has not been found to produce adverse effects (65). In this context, we also evaluated a C/T regimen in CI with a Css of 80 mg/L for optimizing the treatment of C/T-non-susceptible *P. aeruginosa* isolates. Our results showed that this higher dose displays a slight advantage than the currently recommended regimen in CI (Css 45 mg/L).

In summary, our findings showed that C/T in CI achieves a greater overall reduction in bacterial burden than intermittent or extended regimens, particularly in the case of non-susceptible XDR *P. aeruginosa* isolates. C/T in CI at higher Css showed the strongest bactericidal effect, while the administration of suboptimal doses resulted in the emergence of C/T resistance. The correct use of C/T dosing regimens could lead to improve clinical management of *P. aeruginosa* infections caused by XDR isolates.

In vitro studies had some limitations. Regarding, the checkerboard studies, they should be used only as a screening, since it is a static model with fixed time and concentration and with low reproducibility (97,98). Time-kill curves provide time as a dynamic point, but they were lengthened only to 24 h, being not representative of the clinical administration guidelines for the majority of antibiotics (97). Apart from that, antibiotic combinations were studied using fixed concentrations and, since the interaction between antibiotics is dynamic and concentration-dependent (93), the results could vary if other concentrations were analysed. Furthermore, it should be noted that *in vitro* studies cannot examine toxicity, contribution of immune system or the different PK/PD effects occurring at the specific site of an infection.

The one-compartmental chemostat experiments adds drug concentrations as dynamic factor, apart from time. Nevertheless, they were lengthened only 24 h; longer-duration experiments are needed to assess resistance emergence and to represent clinical administration guidelines. Regarding the hollow-fiber infection model, although it has been intended to use concentrations, dosing intervals and experimental durations similar to those expected to apply clinically, toxicity and the diverse PK/PD effects occurring at a specific site of infection cannot be examined, besides the lack of immune system in the role of bacterial killing. However, this absence may be extrapolated easily to immunocompromised patients as well as allow to measure directly the drug antimicrobial activity.

In conclusion, the final purpose of these *in vitro* studies was to approximate an optimized and individualized antimicrobial regimen for each patient through

monitoring antibiotic levels and adjusting antibiotic doses, not only based on the MIC value, but also, on the targeted PK/PD. Moreover, the non-optimization is one of the major implicated causes in antibiotic resistance emergence and is a leading cause of morbidity and mortality worldwide. The *in vitro* data obtained provide a basis for expanding research in this direction and ultimate evaluation in clinical use, in order to increase antibiotic effectiveness with low rates of resistance selection.

6. CONCLUSIONS

- Colistin plus meropenem combination is bactericidal and synergistic against representative isolates of XDR *P. aeruginosa*. It could be a potential option in severe infections caused by *P. aeruginosa* high-risk clones, including carbapenemase-producing isolates and even panresistant isolates.

- The combination of C/T and colistin led to a rapid and sharp decrease in bacterial burden against *P. aeruginosa* isolates, regardless of C/T susceptibility. The combination demonstrated superior synergistic or additive effect against 21 of 24 isolates studied.

- Combination therapy with C/T and colistin would benefit patients with severe *P. aeruginosa* infections with a C/T MIC above the susceptibility breakpoint.

- Monotherapy with colistin resulted in the development of colistin-resistant subpopulations. The combination of colistin plus C/T could prevent resistance development from occurring.

- CZA plus colistin was additive or synergistic in 100% of the CZA-resistant isolates, while CZA plus amikacin and CZA plus aztreonam was additive or synergistic in 85%.

- The combination of CZA plus aztreonam was effective against three of four MBL-carrying *P. aeruginosa* isolates. The addition of aztreonam might overcome CZA-resistance in MBL-bearing strains, being a viable option against MBL-producing *P. aeruginosa* isolates.

- C/T in CI achieved a greater overall reduction in bacterial burden than intermittent or extended dosing regimens against XDR *P. aeruginosa* isolates.

- C/T in CI regimen has demonstrated to be a useful strategy, even against C/T non-susceptible *P. aeruginosa* isolates, but it would be necessary to adjust antibiotic Css.

- The administration of suboptimal C/T Css resulted in the emergence of C/T resistant subpopulation, which showed a deletion in the gene encoding AmpC beta-lactamase.

- Higher C/T Css showed a slight advantage in effectiveness than the currently recommended regimen. It is of particular interest in *P. aeruginosa* isolates with C/T MIC values above 2 mg/L, which have been associated with poor outcomes when treated with a standard C/T dosing regimen.

7. FUTURE LINES OF RESEARCH

After carrying out the presented studies, the line of research will continue focused on analyzing different treatments for XDR *P. aeruginosa* infections.

On the one hand, it is known that combination therapy is a potential therapeutic option for XDR *P. aeruginosa* infections. Existing data support the combination of CZA and aztreonam against class A carbapenemases- and MBL- producing Enterobacterales. However, data about combination against SBL- and MBL- producing *P. aeruginosa* are scarce. A study to compare the efficacy of CZA in combination with aztreonam was analysed against SBL- and MBL- producing XDR *P. aeruginosa* isolates. Isolates were tested by time-kill curves and hollow-fiber infection model. The combination improved the *in vitro* activity of both monotherapies, suggesting that it may be a viable treatment option against SBL- and MBL- producing *P. aeruginosa* isolates. These results were presented at 32nd European Congress of Clinical Microbiology and Infectious Diseases in April, 2022. For future studies, these findings will be validated in a larger collection of SBL- and MBL- producing *P. aeruginosa* isolates.

On the other hand, the use of new antipseudomonal agents could improve the prognosis for patients with greater clinical efficacy. Cefiderocol has a characteristic antibacterial spectrum with a potent activity against resistant Gram-negative pathogens, including *P. aeruginosa*. It has demonstrated promising activity against MBL- producing *P. aeruginosa*.

Future studies will include an analysis of the efficacy of CZA in combination with aztreonam compared to cefiderocol in a representative selection of MBL- producing XDR *P. aeruginosa* isolates.

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