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Comparative in vitro activities of meropenem in combination with colistin, levofloxacin, or chloramphenicol against *Achromobacter xylosoxidans* strains isolated from patients with cystic fibrosis

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ABSTRACT

Objectives: Achromobacter xylosoxidans is an emerging pathogen in cystic fibrosis (CF). Relatively little is known about its clinical impact and optimal management. In the present study, the in vitro bactericidal activities of meropenem, either alone or in combination with colistin, levofloxacin, or chloramphenicol, were assessed using *A. xylosoxidans* strains isolated from CF patients. The synergistic interactions of these combinations were also investigated.

Methods: Minimal inhibitory concentrations (MICs) were determined by microbroth dilution. Bactericidal and synergistic effects of the tested antibiotic combinations were assessed by using the time-kill curve technique.

Results: Based on the time-kill curves, we found that meropenem-colistin combinations have bactericidal and synergistic activities for 24 h against *A. xylosoxidans* strains, both at 1 × MIC and 4 × MIC. Although synergistic interactions were seen with meropenem-levofloxacin combinations, no bactericidal interactions were observed. Additionally, the meropenem-chloramphenicol combinations were found to be neither bactericidal nor synergistic. No antagonism was observed with any combination tested.

Conclusions: This study's findings could have important implications for empirical or combination antimicrobial therapy with tested antibiotics.

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1. Introduction

Achromobacter xylosoxidans, which is an aerobic, nonfermentative Gram-negative bacillus, is widely distributed in aquatic environments and has been detected in the human ear canal, as well as in the gastrointestinal and respiratory tracts [1]. The organism can cause a variety of diseases, including meningitis, bacteraemia, pneumonia, urinary tract infections and nosocomial infections from contaminated environmental reservoirs such as medications, nebulisers and dialysis fluid disinfectants [2–4]. In particular, the rate of diagnosis of colonisation or infection of the airways with *A. xylosoxidans* has increased in cystic fibrosis (CF) patients [5–7]. The predominance of *Achromobacter* infections in these patients has been seen to vary quite widely between populations, with approximations of 16.3–23.9% [8,9]. Besides mobility mechanisms, such as enhanced swarming ability, which is a flagella-dependent type of bacterial motility in low-viscosity liquid and viscous environments, a major virulence factor is its ability to produce and survive in biofilms [10]. Biofilm production is linked with resistance to environmental circumstances by increasing the intimate attachment to surfaces, resistance to phagocytic activity and different host immune factors, shielding from antimicrobial activity and improvement of dissemination across surfaces via bacterial motility. Because *A. xylosoxidans* can serve as a reservoir of horizontal genetic transfer elements commonly involved in spreading antibiotic resistance, the biofilm mode of growth is likely to favour the development of multidrug resistance in CF patients [11,12].

Because the treatment of *Achromobacter* infections can be challenging [13–16], and the effectiveness of empirical treatment for CF-related infections with commonly available antimicrobials is threatened by the development of microbial resistance, enhancing management strategies with existing treatments has played a vital part. Therefore, in the present study, antimicrobial susceptibility and the bactericidal effects of meropenem, levofloxacin and chloramphenicol alone were investigated against *A. xylosoxidans* strains isolated from CF patients. Also, the in vitro bactericidal and synergistic effects of meropenem, combined with colistin, levofloxacin or chloramphenicol, were examined.

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2. Materials and methods

2.1. Bacterial isolates

Twenty-one non–epidemiologically related clinical isolates of *A. xylosoxidans* strains isolated from CF patients and one type strain (LMG 1863T) were obtained from the Department of Clinical Microbiology, Aarhus University Hospital, Aarhus, Denmark. Identification, nrdA allele and pulsed-field gel electrophoresis (PFGE) profiles of clinical strains have been reported [17]. The *Pseudomonas aeruginosa* reference strain ATTC 27853 was used to verify that the MIC values were within the correctness range stated by Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

2.2. Antibiotics

Meropenem trihydrate (AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK), colistin sulphate (Sigma-Aldrich, St. Louis, MO), levofloxacin (Sanofi Pharmaceuticals, Istanbul, Turkey), and chloramphenicol (Deva Pharma, Istanbul, Turkey) were used. Stock solutions of tested antibiotics were prepared in water for injection according to the manufacturer's recommendations at a concentration of 5120 mg/L. The stock solution of colistin and levofloxacin were stored frozen at −80 °C and were used within 6 months. Meropenem and chloramphenicol solutions were prepared on the day of use.

2.3. Media

Cation-adjusted Mueller Hinton Broth (CAMHB) was prepared daily by adding 25 mg/L calcium to liquid and 12.5 mg/L magnesium to Mueller Hinton broth (Oxoid, Basingstoke, UK). This medium was used for MIC and time-kill assay studies. Tryptic soy agar (Oxoid) was used for colony counts.

2.4. Determination of MIC

MICs were determined by the microbroth dilution technique described by the CLSI guidelines [18]. Serial twofold dilutions, ranging from 32 to 0.03 mg/L for meropenem and colistin, and from 256 to 0.25 mg/L for levofloxacin and chloramphenicol, respectively, were prepared in fresh CAMHB 96-well microtiter plates. The inoculum was prepared with a 4- to 6-h broth culture. Each bacterial strain was spectrophotometrically adjusted to OD_{600} = 0.12–0.13 (corresponding to ∼1 × 10^8 CFU/mL) and further diluted in CAMHB to obtain a final concentration of 5 × 10^5 CFU/mL in the test tray. The trays were placed in plastic bags to avoid evaporation, incubated at 37 °C for 18–20 h, and visually inspected for growth. CLSI interpretative criteria for susceptibility of *P. aeruginosa* ATCC 27853 were used.

2.5. Determination of time-kill curves

The time-kill curve assay was performed to observe the dynamics of the bactericidal activity of meropenem, colistin, levofloxacin and chloramphenicol alone on five isolates representing different susceptibility patterns at 1 × MIC and 4 × MIC. Also, time-kill assays were performed to evaluate the concentration-dependent bactericidal and synergistic activity of meropenem combined with colistin, levofloxacin and chloramphenicol at 1× and 4 × MIC following the National Committee for Clinical Laboratory Standards (NCCLS) [19]. Time-kill assays were sampled for colony counts at 0, 2, 4, 6 and 24 h. This method detected 20 CFU/mL at the lower limit. According to this method, bactericidal activity was defined as a ≥3-log10-unit decrease in the original inoculum within 24 h. Synergy and antagonism are defined as a ≥2 $\log_{10}$ decrease or increase, respectively, in CFU/mL at 24 h for the antibiotic combination compared with the most active antimicrobial agent alone. The additive effect was described as <2 $\log_{10}$ increase or decrease in colony count at 24 h by the combination compared with the most active single antimicrobial alone.

3. Results

The MICs of meropenem, colistin, levofloxacin and chloramphenicol for the 22 strains of *A. xylosoxidans* are shown in Table 1. According to the CLSI criteria, the tested antibiotics' susceptibility rates for tested 22 strains were ranked as follows: meropenem > levofloxacin > colistin > chloramphenicol. On a weight basis, meropenem was the most active antimicrobial agent tested with the MICs. According to the CLSI breakpoint, the results revealed that 91%, 77%, 64% and 59% of tested strains were susceptible to meropenem (MIC50/90 = 0.125/4 mg/L; 2/22), to levofloxacin (MIC50/90 = 2/8 mg/L; 17/22), to colistin (MIC50/90 = 2/16 mg/L; 14/22), and chloramphenicol (MIC50/90 = 32/64 mg/L; 9/22), respectively. All tested strains were resistant or moderately susceptible to chloramphenicol (Table 1).

The strains used in time-kill cure studies were AX-1, AX-2, AX-3, AX-4 and AX-5; they were chosen for their different susceptibility patterns. AX-1 was intermediate to colistin and chloramphenicol. AX-2 was intermediate to chloramphenicol. AX-3 was resistant to colistin and intermediate to chloramphenicol. AX-4 was intermediate to colistin and resistant to chloramphenicol. AX-5 was resistant to levofloxacin and chloramphenicol, whereas all other strains were susceptible to them (Table 2).

The results of the time-kill curve study indicated that colistin was rapidly bactericidal against AX-1 at 1 × MIC and 4 × MIC as well as against AX-3 and AX-4 only at 4 × MIC within 6 h (Fig. 1). Viable counts of the colistin-treated (1 × MIC or 4 × MIC) culture could rapidly drop by >3-$\log_{10}$ units at 6 h but not for 24 h, whereas the untreated control culture continued to grow. Otherwise, both at 1 × MIC and 4 × MIC, meropenem, levofloxacin or chloramphenicol alone did not display any bactericidal effect against tested strains within 24 h (Table 2).

The findings of the tested antibiotic combinations by time-kill assay indicated that meropenem-colistin combinations showed synergistic effects on two of five *A. xylosoxidans* strains at 1 × MIC and three of five strains at 4 × MIC (Fig. 1). This combination is not only significantly synergistic against *A. xylosoxidans* strains but also, at 4 × MIC, it reduced live *A. xylosoxidans* cells to >3 $\log_{10}$ CFU/mL. Meropenem-colistin produced bactericidal effects against one of five strains at 1 × MIC and four of five strains at 4 × MIC. Moreover, when meropenem and colistin were used in combination at 4 × MIC, the bactericidal effect lasted up to 24 h. On the other hand, a meropenem-levofloxacin and meropenem-chloramphenicol combination had no bactericidal effect. The meropenem-levofloxacin combination exhibited a synergistic effect against one

Table 1
In vitro activities of tested antibiotics against 22 *Achromobacter xylosoxidans* strains.

Strain	MIC (mg/L)			Susceptibility (*n*, %)		
	MIC range	MIC_{50}	MIC_{90}	S	I	R
MEM	0.06–32	0.125	4	18 (82)	2 (9)	2 (9)
CST	0.25–64	2	16	11 (50)	3 (14)	8 (36)
LVX	1–16	2	8	13 (59)	4 (18)	5 (23)
CHL	16–128	32	64	0	13 (59)	9 (41)

Note: CLSI breakpoints for P. aeruginosa for susceptibility and resistance to meropenem are ≤2 mg/L and ≥8 mg/L, for colistin are ≤2 mg/L and ≥8 mg/L, for levofloxacin are ≤2 mg/L and ≥8 mg/L and for chloramphenicol (for other non-Enterobacteriaceae) are ≤8 mg/L and ≥32 mg/L.
CHL, Chloramphenicol; CST, colistin sulphate; LVX, levofloxacin; MEM, meropenem.

Table 2
Bactericidal and synergistic effects of tested antibiotic combinations against five *Achromobacter xylosoxidans* strains.

MIC (mg/L)					Combination effects											
					MEM-CST				MEM-LVX				MEM-CHL			
Strain	Antibiotics				1 × MIC		4 × MIC		1 × MIC		4 × MIC		1 × MIC		4 × MIC	
	Mem	Cst	Lvx	Chl	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A
AX-1	0.125	4	2	16	+	S	+	S		A		A		A		A
AX-2	0.125	0,5	2	16		A	+	A		A		A		A		A
AX-3	0.06	8	2	16		A	+	S		A		S		A		A
AX-4	0.125	4	1	32		S		A		A		A		A		A
AX-5 (LMG1863 T)	0.5	2	8	32		A	+	S		S		S		A		A
PA	0.5	1	1	128												

A, Additive, <2-$\log_{10}$ increase or decrease in colony count at 24 h by the combination compared with the most active single antimicrobial alone; BCT, bactericidal effect, ≥3-$\log_{10}$ unit decrease in the original inoculum within 24 h; AX, *Achromobacter xylosoxidans*; CHL, chloramphenicol; CST, colistin sulphate; LVX, levofloxacin; MEM, meropenem; PA, *Pseudomonas aeruginosa* ATCC 27853 (reference strain); S, synergy: ≥ 2 $\log_{10}$ decrease in CFU/mL at 24 h for the antibiotic combination compared with the most active antimicrobial agent alone.

of five strains at 1 × MIC and two of five strains at 4 × MIC. Additionally, meropenem-chloramphenicol combinations tested were only found to be additive. No antagonism was observed in any combination used in the study (Table 2).

4. Discussion

Data in the literature strongly suggest that in patients who lack natural defence mechanisms against infection, better clinical results can be achieved by treating illness with two drugs to which the pathogenic organism is sensitive rather than with only one. Moreover, there is evidence that the use of antibiotic combinations found to be synergistic in vitro is associated with a better clinical outcome in patients with decreased defence mechanisms [7–9,20]. Because in vitro synergy or, at least, an additive effect between antibiotics in vitro, is likely to have clinical significance, the use of synergistic drug combinations should be continued as empirical therapy in severely neutropenic or otherwise compromised patients. Combination therapy can play an essential role in the management of severe *A. xylosoxidans* infections and has also been recommended for the treatment of *A. xylosoxidans* pulmonary exacerbations in cases of CF [21,22].

In previous studies, Mathy et al. [22] and Duggan et al. [23] reported the synergistic effects of some antibiotic combinations against this bacterium, but this has not yet been reported for colistin combined with meropenem. Because well-defined pharmacodynamic parameters have not yet been firmly established for *A. xylosoxidans*, our study investigated meropenem, colistin, levofloxacin and chloramphenicol alone for their bactericidal effects at 1 × MIC and 4 × MIC within 24 h. Also, meropenem, which is the first-choice carbapenem for *A. xylosoxidans* antibiotic therapy [24], was combined with colistin, levofloxacin or chloramphenicol. These combinations were then investigated for their bactericidal and synergistic effects at 1 × MIC and 4 × MIC within 24 h. According to our findings, although meropenem prevented an increase in the number of live bacteria for 6 h, it did not display any bactericidal effects against any tested strains at 1 × MIC and 4 × MIC within 24 h. However, the time-kill curves showed that when meropenem was used in combination with colistin, which has a detergent-like mechanism and is a second-line antibiotic in *A. xylosoxidans* therapy [24–26], the combination produced bactericidal activity against meropenem-susceptible and colistin-susceptible or -resistant strains, even when the substances alone were not bactericidal for 24 h. The presence of the bactericidal (>3 $\log_{10}$ killing) effect of the meropenem–colistin combination may allow this combination to be used in severe infections requiring rapid microbial killing. The results of the present in vitro trial provide initial evidence that meropenem used in combination with colistin sulphate may produce clinically relevant synergy against colistin-susceptible or -resistant *A. xylosoxidans* at both 1 × MIC and 4 × MIC. This situation is in agreement with the results on other Gram-negative, nonfermentative bacteria, such as *Acinetobacter baumannii* or *P. aeruginosa*, and Enterobacteriaceae, such as *Klebsiella pneumoniae* [23,26]. This antibiotic alone at 4 × MIC exhibited a more bactericidal effect than at 1 × MIC. The result indicated that it might be useful to use meropenem in combination with colistin sulphate to produce a rapid enhancement of bactericidal activity and prevent or delay the emergence of resistance. Additionally, because this combination could produce synergistic activity on two strains at 1 × MIC and three strains at 4 × MIC, it might be useful to gain synergistic interaction against this organism.

It is essential to investigate useful antibiotic combinations to reduce bacterial burdens rapidly such as quick mortality and obtain clinical success and repression of amplification of less sensitive bacterial subpopulations [27–30]. For these purposes, combinations of a β-lactam and an aminoglycoside or fluoroquinolone have been investigated for many microorganisms. CLSI recommends levofloxacin as a second-line reporting antibiotic in combination therapy of *A. xylosoxidans* [24–26]. Although intrinsic fluoroquinolone resistance could be seen in this antibiotic class, the use of fluoroquinolones is preferred to gain their in vitro synergistic effects, as previously reported [27–31]. Furthermore, fluoroquinolones generally produce significant in vitro bactericidal and synergistic effects against Gram-negative strains. Similar to previous studies [27–29] of other Gram-negative bacteria, in the present study, the meropenem-levofloxacin combination at 1 × MIC produced an average of a 1-$\log_{10}$ reduction effect against tested *A. xylosoxidans*, and the results were prolonged in a concentration-dependent manner (Fig. 1). However, at 4 × MIC, the meropenem-levofloxacin combination did not show a bactericidal effect. It accomplished an average of a 2-$\log_{10}$ killing effect throughout 24 h, and this combination produced synergy against one strain (AX-3) at 1 × MIC and against two strains (AX-3 and AX-5) that were susceptible or resistant to levofloxacin at 4 × MIC. Also, combination therapy with meropenem and levofloxacin provides additive effects against the other *A. xylosoxidans* strains. Thus, it could be stated that the combination might be useful to obtain synergistic interaction or an additive effect against this organism.

In CF, the treatment of bacterial infections is often complicated by resistance to the aminoglycosides and variable susceptibility to β-lactams and quinolones. For this reason, therapy should be directed by susceptibility testing [32]. Chloramphenicol is used alone or in combination with other antibiotics to treat lung infections caused by specific bacteria, such as *P. aeruginosa* and *A.*

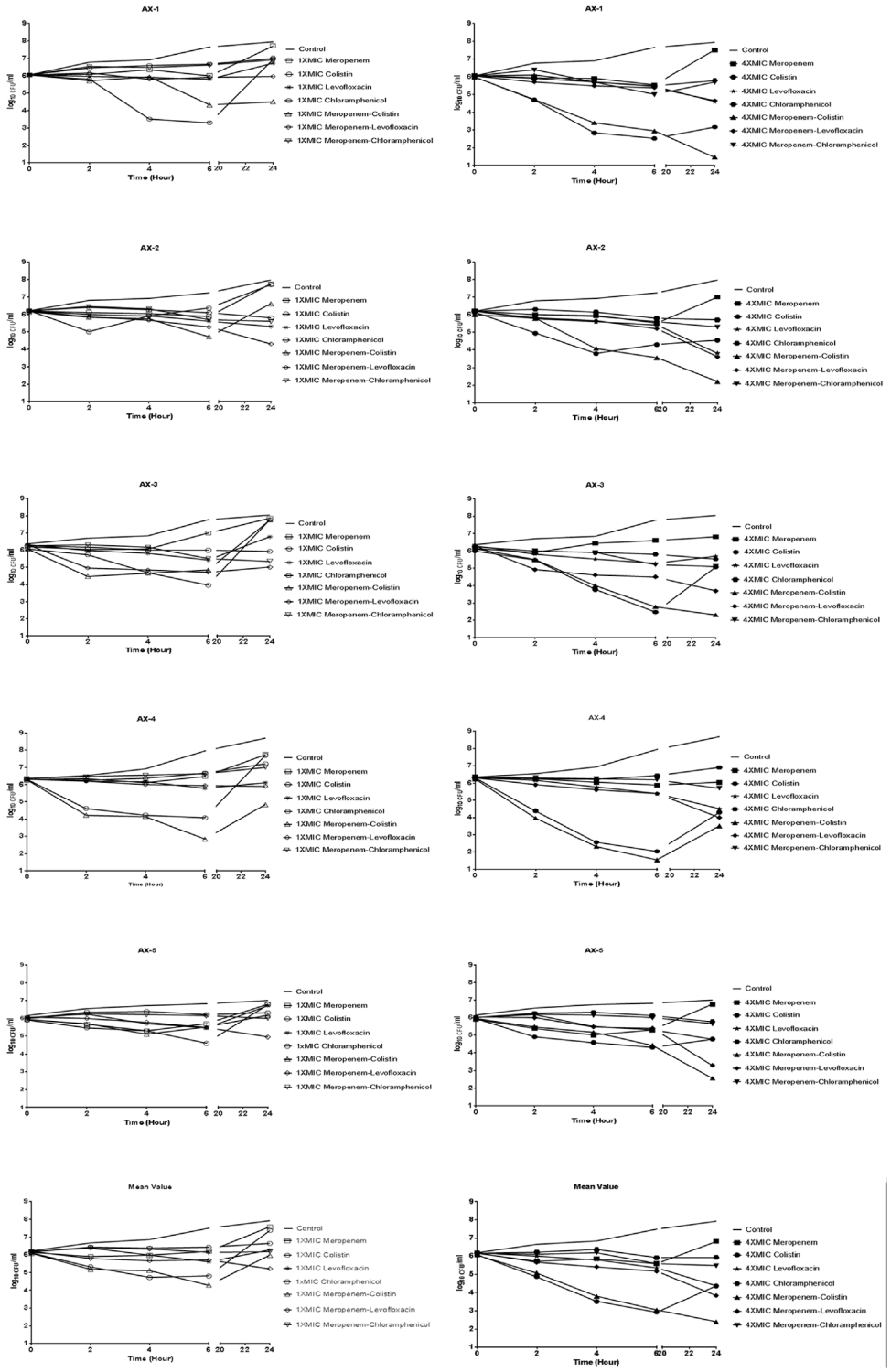


Fig. 1. Time-kill curve analysis for meropenem, colistin, levofloxacin, chloramphenicol and meropenem in combination with colistin or levofloxacin or chloramphenicol against five *Achromobacter xylosoxidans* strains.

xylosoxidans. In a previous study by Samian et al. [13], the most active additive or synergistic combinations were chloramphenicol plus minocycline, ciprofloxacin plus imipenem and ciprofloxacin plus meropenem. In our study, chloramphenicol alone did not exhibit any bactericidal effects against strains at the tested MICs. Instead, it maintained approximately the same number of viable bacteria as starter inoculum (1×106 CFU) at $1 \times$ MIC and $4 \times$ MIC for 24 h.

Similarly, meropenem-chloramphenicol combinations did not show any bactericidal effects against the tested strains. Combinations that include chloramphenicol demonstrated horizontal time-kill curves at $1 \times$ MIC and $4 \times$ MIC for 24 h against *A. xylosoxidans*; the combinations prevented bacterial growth regardless of concentration. Moreover, combinations that included chloramphenicol only displayed additive effects against all strains at 24 h. Our findings showed that chloramphenicol alone or used in

combination was a bacteriostatic option for *A. xylosoxidans* therapy.

In conclusion, this study reveals that meropenem used in combination with colistin sulphate or levofloxacin could produce clinically relevant synergy against *A. xylosoxidans*, and a meropenem-colistin combination increases the potency of killing activity. To our knowledge, this study is the first investigation in which a combination of meropenem with colistin has demonstrated promising bactericidal and synergistic activity against *A. xylosoxidans* strains, including colistin-intermediate or colistin-resistant strains. This combination should be further evaluated in a clinical trial.

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Conflict of interests

The authors declare that there are no conflicts of interest.

Ethical approval

This was not required.

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