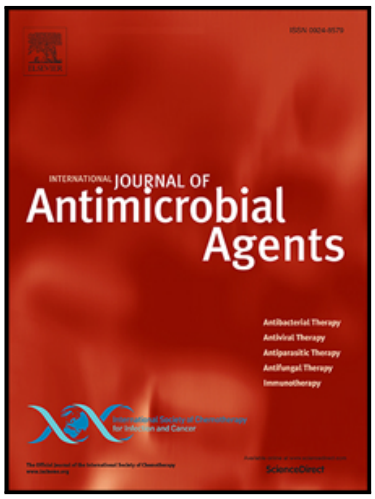

Serum bactericidal activity of colistin and azidothymidine combinations against mcr-1 positive colistin-resistant Escherichia coli

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Highlights

- Concentrations from phase-1 study with CMS and AZT in combination with loading and maintenance dose
- Bactericidal activity in serum spiked with colistin and AZT concentrations alone or combined
- Combination of colistin with AZT increased serum bactericidal activity
- Dosage of 2 MIU CMS + AZT 100mg q12 sufficient to treat infection with colistin-susceptible strains
- for infections with colistin-resistant strains the dosage should be higher

Serum bactericidal activity of colistin and azidothymidine combinations against *mcr-1* positive colistin-resistant *Escherichia coli*

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Running title: serum bactericidal activity of colistin and azidothymidine combinations

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Abstract

To examine the serum bactericidal activity of colistin-sulphate (CS) and azidothymidine (AZT) combinations, time-kill curves were performed in native and heat-inactivated human serum with five colistin-resistant and four colistin-susceptible Gram-negative strains. The serum samples were spiked according to the median and minimum plasma peak concentrations measured in a phase 1 clinical study, in which seven healthy subjects received 3-times (q12) 1h-IV-infusions of 4, 2 and 2 million international units (MIU) colistin-methanesulfonate (CMS) co-administered with 200, 100 and 100 mg AZT, respectively. This trial was performed to assess the pharmacokinetics and safety of CMS/AZT-combination therapy.

Minimal bactericidal concentrations of CS in native, but not heat-inactivated serum, were strongly reduced compared to Mueller-Hinton-Broth for all tested Enterobacteriaceae, except one colistin-resistant (serum-resistant) strain. For colistin-susceptible strains, the minimum CS concentration after 2 MIU CMS dosage was already bactericidal in native and heat-inactivated serum. Median, but not minimum, CS concentrations after 2 MIU CMS dosage were sufficient to kill the serum-resistant, colistin-resistant *E.coli* strain in native serum. In heat-inactivated serum, even the median CS concentration after 2 MIU CMS dosage was not bactericidal for all colistin-resistant strains. In general, combinations with AZT accelerated killing of colistin-resistant *E.coli* or showed bactericidal activity even if the substances alone were not bactericidal. Thus, the combination with AZT potentiates the bactericidal effect of colistin against colistin-resistant *E.coli* strains. Although the dosage of 2 MIU CMS plus AZT may be sufficient to treat infections with colistin-susceptible strains, for infections caused by colistin-resistant *E.coli* the dosing should be further optimized.

Keywords: Colistin, Azidothymidine, combination therapy, serum bactericidal activity

Introduction

Gram-negative bacteria, especially Enterobacteriaceae, are the major cause of both community and hospital-acquired urinary tract infections (UTI), which is worsened by the continuous antibiotic resistance emergence and a shortage of new antibiotics under development. The number of antimicrobial agents to medicate infections caused by antibiotic-resistant Enterobacteriaceae is limited, and the polymyxins such as colistin are considered the last resort treatment [1].

Two different forms of colistin are available for clinical use, colistin sulphate (CS) and colistin methanesulfonate (CMS). CS is used for treatments of topical and gastrointestinal infections but is not used systemically as it causes undesirable side effects such as irritation at subcutaneous or intramuscular injection sites. CMS is preferred for parenteral and aerosol therapies [2] since it causes fewer adverse reactions than CS. In aqueous solutions, CMS undergoes hydrolysis to form a complex mixture of partially sulfomethylated derivatives as well as colistin [2]. Unfortunately, resistance to colistin is rapidly emerging [3]. The first discovery of the transmissible plasmid-mediated colistin resistance encoded by the mobilized colistin resistance (*mcr-1*) gene in China [4] rapidly followed by numerous reports of the plasmid-borne *mcr-1* gene in animals, food and clinical samples worldwide [5]. Most importantly, the *mcr-1* colistin resistance was clearly shown to coexist with other multiple drug resistant (MDR) genes, highlighting the possibility that pan-drug resistant bacterial strains are emerging [6]. Therefore, a different strategy is urgently required to preserve, restore or enhance the therapeutic potencies of existing antibiotics against resistant bacteria. Such strategies include repurposing marketed drugs, as these have known toxicology and pharmacology profiles, to interact synergistically with existing antibiotics.

Azidothymidine (AZT, zidovudine) is an antiretroviral drug, which is used in combination with other antivirals to prevent and to treat human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) [7]. AZT acts antibacterial against Enterobacteriaceae [8]. Furthermore, a synergistic antibacterial activity between AZT and gentamicin or colistin was shown for Enterobacteriaceae strains recently [9, Hu et al. submitted, unpublished data].

The aim of this study was to determine the serum bactericidal activity of CS and AZT combinations against five colistin-resistant *Escherichia coli* strains as compared to colistin-susceptible *E. coli, Acinetobacter baumannii and Pseudomonas aeruginosa* strains using concentrations of both drugs obtained from a phase 1 study [10].

Material & Methods

Ethical approval and conduct

This *ex-vivo* annex trial is part of a randomized, double-blinded, placebo-controlled dose escalation study in healthy volunteers receiving multiple doses of IV CMS co-administrated with AZT performed in the phase 1 unit of the QuotientSciences, Nottingham, UK. The study was performed in accordance with ethical principles that have their origin in the Declaration of Helsinki, and are consistent with International Conference on Harmonization/Good Clinical Practice. The phase 1 study was registered with the European Clinical Trial database EudraCT (#2016-002767-34).

Study design and plasma concentrations

Detailed safety information, laboratory test results and pharmacokinetic evaluations of this phase 1 study are presented elsewhere [10]. Seven volunteers received 3 times (q12) 1 h IV-infusions of 4, 2 and 2 million international units (MIU) CMS co-administered with 200, 100 and 100 mg AZT, respectively.

Plasma samples were collected at different time points, were frozen immediately to -80 °C and kept at -80°C until analysis no longer than 4 weeks. Samples were analysed by validated LC-MS/MS method [11]. Maximum, median and minimum plasma concentrations of colistin and AZT obtained at the end of the 1 h IV infusion of the 1st, 2nd and 3rd dose are presented in table 1. Concentrations of colistin were expressed in colistin base, whose molecular weight slightly differs from CS (colistin base – 1155.5; CS – 1400.7; corresponding to colistin B [12-13]). Therefore, we converted the obtained colistin base concentrations to CS (factor 1.212), which were then used for spiking the serum in the time-kill curve determinations (table 1).

Bacterial strains

Five clinical isolates harbouring the colistin-resistance gene *mcr-1* (table 2) were used. In addition two isolates from urine of patients with UTI, *P. aeruginosa* 1640801 and *A. baumannii* CDH102, both showing carbapenem-resistance and the reference strains, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were included in this study as colistin-susceptible strains.

Determination of minimal inhibitory and bactericidal concentrations (MIC/MBC)

MIC/MBC determinations were performed according to the CLSI- and EUCAST-Standards [15-17]. Broth microdilution assay was used for determination of the MIC/ MBC of CS and AZT.

Cation-adjusted Mueller Hinton broth (CAMHB) (Sigma Aldrich) and human serum, originated from pooled plasma collected from male donors of the AB serotype, (Sigma Aldrich) were used. For heat-inactivation, the serum was incubated for 30 min at 56°C. Two-fold serial dilutions of media CS were prepared in 96-well plates (polystyrene, Greiner Bio-One, Frickenhausen, Germany). Final inocula, which were confirmed by actual plating, ranged from 2 – 8 x 10^5 CFU/ml. After incubation at 37°C for 20 ± 2 h in ambient air the optical density (OD) at 600 nm was measured using a Multiskan GO plate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The MIC was defined as the lowest concentration inhibiting growth (OD_{600} ≤ 0.1). For determination of MBCs, in a second step 3 µl from each well of the overnight incubated 96-well plate were transferred onto IsoSensitest agar supplemented with 5% blood (Oxoid, Wesel, Germany) using an one-time inoculator (Dr. Brinkmann Floramed, Nürtingen, Germany). The plates were incubated overnight at 37°C. The number of colonies, subsequently grown, was used to determine the bactericidal endpoint. MBC was defined as a > 99.9% (> 3-log) reduction of the initially inoculated colony counts. All determinations were repeated at least three times.

Time-kill curve

Time-kill curve analyses were performed by culturing the test strains in native or heat-inactivated serum in the presence of CS and AZT, alone and in combination. CS and AZT quantities corresponding to median and/or minimum plasma concentrations 1 h after 1st and 3rd dose obtained from the phase 1 study were used. Bacterial strains (inoculum ~ 5*10^5 CFU/ml) were inoculated in native or heat-inactivated serum in polycarbonate tubes (Sarstedt, Nümbrecht, Germany) and were allowed to equilibrate for 30 min at 37°C. Earlier studies indicated much lower sticking of colistin to polycarbonate than to polystyrene (data

not shown). CS and AZT, alone or in combination, were added and tubes were incubated at 37°C at 180 rpm. Samples were taken for determination of viable counts at 0, 15, 30, 60, 120, 240, 360 and 1440 min after the substances have been added. Serial dilutions were prepared in sterile PBS and plated onto MHB-agar plates. The plates were incubated at 37°C for 20 h, when the numbers of colonies were determined. Kill curves were constructed by plotting the log10 CFU/ml over 24 h. The detection limit was ~ $1*10^2$ CFU/ml. Time-kill curves were performed once for each strain.

Results

Minimal inhibitory and bactericidal concentrations

The MICs of CS measured in CAMHB ranged from 4 to 8 mg/l for the *mcr-1* positive strains, which are classifying them as colistin resistant according to EUCAST criteria [18]. The MIC values of CS in CAMHB for *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *P. aeruginosa* 1640801 and *A. baumannii* CDH102 ranged from 0.375 -1 mg/l classifying them as colistin susceptible (table 3). MBC values of CS in heat-inactivated serum are almost equal to those in CAMHB. In contrast, MBC values in native serum were considerably lower for *E. coli* ATCC 25922 and for the *mcr-1* positive *E. coli* strains except for *E. coli* Af49. For *P. aeruginosa* ATCC 27853, *P. aeruginosa* 1640801 and *A. baumannii* CDH102 MBCs values in native serum only differ in one dilution step compared to CAMHB. *E. coli* Af40, Af49, CDF1 and CDF8 as well as *P. aeruginosa* ATCC 27853, *P. aeruginosa* 1640801 and *A. baumannii* CDH102 showed MBC values of AZT between 8 - >16 mg/l in native serum, whereas MBC values of *E. coli* ATCC 25922 and *E. coli* Af23 were 0.25 mg/l and 0.5 mg/l, respectively. The MBC values of AZT in heat-inactivated serum ranged between 32 - >32 mg/l for all strains tested.

Time-kill curve

To determine the serum antibacterial activity time-kill curve experiments were performed in native and heat-inactivated human serum spiked with CS and AZT, alone and in combination, using median and minimum plasma concentrations obtained 1 h after 1st and 3rd dose (table 1).

Median 1st and 3rd dose concentrations of CS were sufficient to kill *E. coli* Af49 in native serum, whereas minimum 3rd dose concentration just led to a slightly delayed growth (figure 1). Adding AZT alone reduced bacterial numbers in a concentration-dependent manner for up to 6 h with a regrowth afterwards. Combination of CS with AZT led to complete killing of *E. coli* Af49 after at least 6 h for all concentrations tested in native serum (figure 1). For all other colistin-resistant and susceptible strains tested, the minimum 3rd dose concentration of CS was sufficient to kill the bacteria within 0.25 h and 4 h in native serum. It was also observed, that native serum by itself had an inhibitory or even a bactericidal effect in the first few hours for some of the tested strains (figure 2-3).

Since a bactericidal activity of native serum has been shown to be heat-labile [19], time-kill curve experiments were also performed in heat-inactivated serum. In heat-inactivated serum, the minimum 3rd dose concentration of CS was not sufficient to kill any of the colistin-resistant strains but was adequate to eliminate all colistin-susceptible strains tested within 2 h (figure 4-5). AZT barely affected the bacterial growth at this concentrations. Combinations of CS with AZT were not beneficial for colistin-susceptible strains but had some bacteriostatic activities against colistin-resistant strains (figure 4-5). Three of the five colistin-resistant strains were at least restricted in growth or even killed by the median 3rd

dose concentration of CS in heat-inactivated serum. Combinations of AZT with CS showed bactericidal or at least bacteriostatic activities for all strains tested (figure 4). The median 1st dose concentration of CS was bactericidal or at least bacteriostatic for all colistin-resistant *E. coli* in heat-inactivated serum. Combination of AZT with CS accelerated the killing of the bacteria or showed bactericidal activity at all as compared to the individual substances alone (figure 4).

Discussion

This *ex-vivo* annex study was carried out as part of a phase 1 study in healthy volunteers [10]. The drug combination was well tolerated, with only transient and manageable gastrointestinal events with mild severity seen in this subgroup of healthy volunteers. The trial evaluated bactericidal activity of plasma concentrations obtained during IV-treatment with CMS in combination with AZT. The targeted bacteria were *mcr-1* positive colistin-resistant *E. coli* strains, which were compared to colistin-susceptible strains.

The median MIC and MBC of colistin in CAMHB for the four *mcr-1* positive *E. coli* strains ranged from 8-32 mg/l classifying them as resistant according to EUCAST criteria [18]. MBC values of colistin in heat-inactivated serum were comparable to those in CAMHB. In contrast, MBC values in native serum were noticeably lower for the *E. coli* but not for *P. aeruginosa* and *A. baumannii* strains. Furthermore, native serum on its own negatively affects the growth of some of the tested strains. The complement system in the human serum could eliminate bacteria by the formation of membrane-attack-complexes, which increase bacterial cell wall permeability [20]. The complement activity is heat-labile [19], which can be confirmed by comparison of the control sample growth in native or in heat-inactivated serum in our time-kill curve data. In these serum-sensitive strains, weakening the bacterial

membrane integrity by the complement system, even without killing the bacteria, could enhance the effectiveness of colistin. By adding 20% human serum to broth, a synergism between serum and the antibacterial activity of colistin was shown previously [21]. Nevertheless, serum-resistant strains, such as *E. coli* Af49 and *A. baumannii* CDH102, which can grow normally in native serum, showed comparable MBCs in native serum and CAMHB.

For *in vitro* kill-curve assays the measured CB concentrations were converted to CS concentrations using a factor of 1.212, corresponding to the differences in molecular weights of CB B and CS B. Since colistin is a mixture of colistin A and B, molecular weights corresponding to colistin A or an average of A and B (CB – 1169.5/ CS – 1414.7 or CB – 1162.5/ CS - 1407.7 [12-13]) could have been used as well. The conversion factors, however, would differ by only ≤ 0.25%, corresponding to a range of 10-20µg/l, which is negligible.

Time-kill curve assays showed that the minimum plasma concentrations of colistin (1.26 mg/l) after 2 MIU CMS dosage reached at least minimal bactericidal concentrations for colistin-susceptible and carbapenem-resistant strains. Due to the human serum's synergistic activity, even minimum CS concentrations after 2 MIU dosage were sufficient to kill the serum-sensitive colistin-resistant strains in native serum. In contrast, inactivating the heat-labile complement system means that even the median CS plasma concentration after 4 MIU CMS dosage (4.91mg/l) was not able to kill all colistin-resistant strains. Thus, it can be assumed that plasma concentrations of CS obtained after a 4 MIU dosage of CMS may not be sufficient to kill serum-resistant colistin-resistant bacteria. Nevertheless, the combination with AZT accelerated killing of the bacteria or acted bacteriostatically/ bactericidally when CS alone was not bactericidal within 4 h or even at all.

The antibacterial activity of AZT can be traced back to the inhibition of the replicative DNA-synthesis after incorporation of azidothymidine-triphosphate [7]. This effect is often only

temporary. Mutations resulting in a lack of thymidine kinase lead to an increased resistance against azidothymidine [7]. AZT resistant strains may emerge also *in vivo*, but no cross-resistance to other antimicrobial agents has been seen with AZT [9, 22]. AZT did not affect the growth of *Pseudomonas* and *Acinetobacter* strains as these species often naturally lack a thymidine kinase [23-24]. Furthermore, the combination with colistin was not conducive to the killing of these strains.

Since medium CS plasma concentrations reached after 1h-IV-infusions of 4 MIU CMS are not sufficient to kill all colistin-resistant strains, the dosage of colistin may need to be increased as one option. Although nephrotoxicity associated with colistin is not as toxic as originally thought [25-26], an adverse effect must still be considered when administering increasing colistin dosages. This risk of nephrotoxicity did not appear to be related to daily doses of CMS, but rather to the total cumulative dose [27]. The risk of nephrotoxicity, although mostly reversible, could even be increased by factors such as concomitant use of a calcineurin inhibitor, hyperbilirubinemia, and hypoalbuminemia [25-26]. In contrast, nephrotoxicity is not described for AZT [28]. Therefore, as an alternative to increasing colistin dosages, combination therapy with lower CMS dose combined with an optimized AZT dosage should be developed further.

Conclusion

This ex-vivo annex study provided evidence that a combination of colistin with AZT potentiates the bactericidal effect of colistin against colistin-resistant *E. coli* strains. This result needs to be confirmed in human studies. Although AZT and colistin plasma concentrations reached after one-hour infusion of 2 MIU CMS combined with 100 mg AZT

are sufficient for the treatment of colistin-susceptible, even carbapenem-resistant, strains, the dosage should be further optimized for infections caused by colistin-resistant *E. coli.*

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Patrice Nordmann, University of Fribourg and IHMA Europe Sàrl kindly provided the *mcr-1* positive *E. coli* strains and the *P. aeruginosa* 1640801 strain, respectively**.**

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Declarations

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Competing Interests: ML: Collaborator – Helperby Therapeutics. KGN: Investigator - Enteris Biopharma; Scientific Advisor (Review Panel or Advisory Committee) - Bionorica, Enteris Biopharma, Helperby Therapeutics, Leo Pharma, MerLion, MSD Sharp & Dohme, OM Pharma, Paratek, RosenPharma, Roche, Zambon; Speaker's Bureau - Bionorica, Daiichi Sankyo, Leo Pharma, OM Pharma, Rosen Pharma, Zambon. YH: Grant Investigator – Helperby Therapeutics. AC: Shareholder (excluding diversified mutual funds) - Helperby Therapeutics. FMEW: Collaborator – Helperby Therapeutics; Investigator - Enteris BioPharma; Scientific Advisor (Review Panel or Advisory Committee) - Bionorica, Enteris BioPharma, Helperby Therapeutics, Leo Pharma, MerLion, OM Pharma, Rosen Pharma, Achaogen, AstraZeneca, Janssen, MSD, Shionogi, Pfizer.

Ethical Approval: Not required

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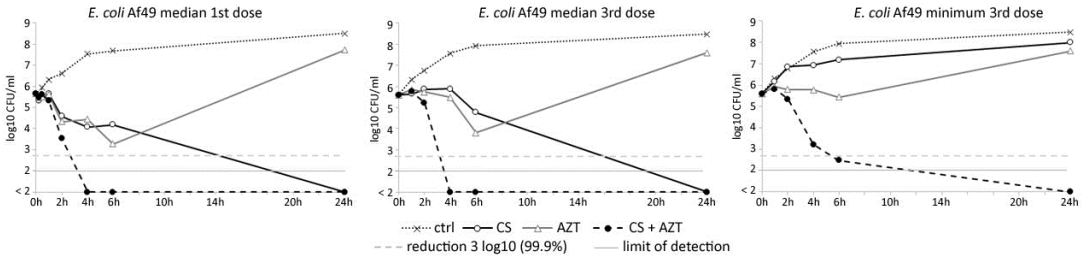

Figure 1 Time-kill curves in native serum with *E. coli* Af49

Bacteria were inoculated with $5*10^5$ CFU/ml in native serum. After adding colistin sulphate (CS) and azidothymidine (AZT) alone or in combination according to the median and minimum plasma concentrations after 1st and/or 3rd dose (1h after IV infusion) bacteria were incubated at 37°C and 180rpm. Ctrl – control without any addition

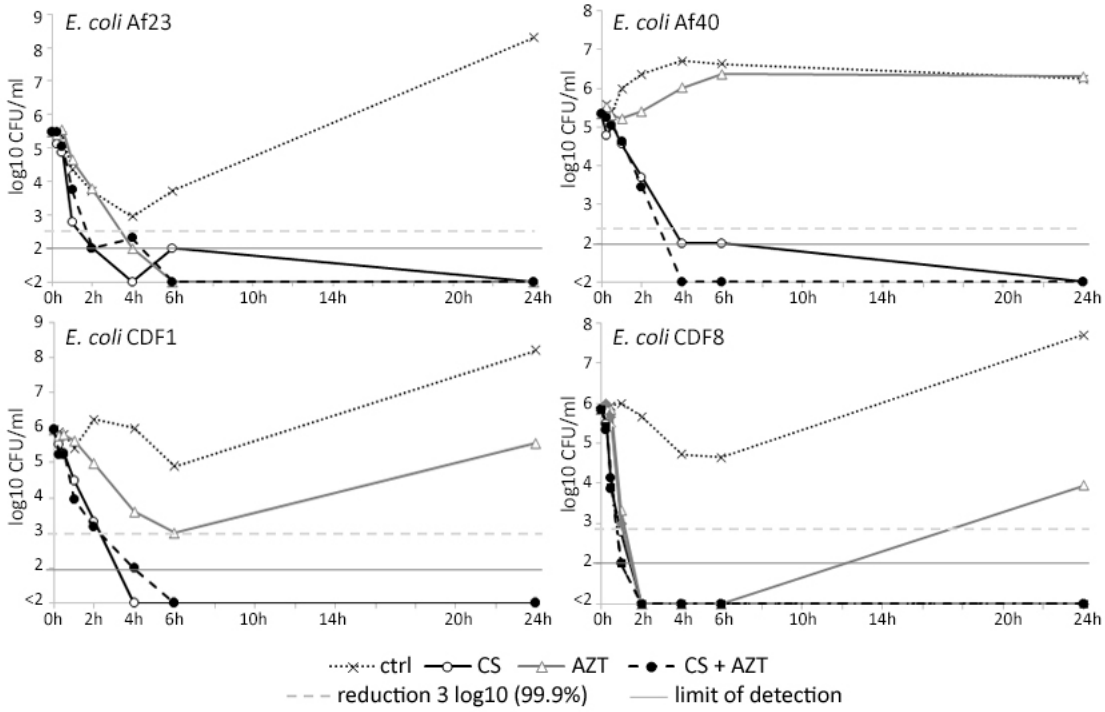

Figure 2 Time-kill curves in native serum of colistin-resistant *E. coli* strains using minimum 3rd dose concentrations

Bacteria were inoculated in native serum. After adding colistin sulphate (CS) and azidothymidine (AZT) alone or in combination according to the minimum plasma concentrations after 3rd dose (1h after IV infusion) bacteria were incubated at 37°C and 180rpm. Ctrl – control without any addition

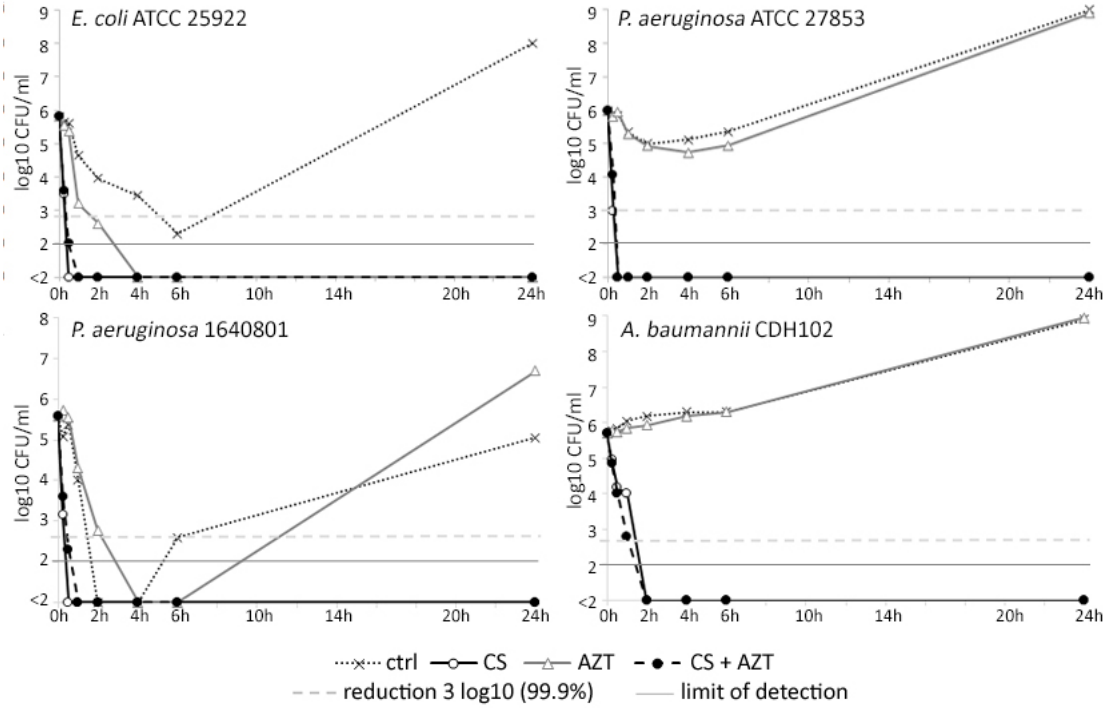

Figure 3 Time-kill curves in native serum of colistin-susceptible strains using minimum 3rd dose concentrations

Bacteria were inoculated in native serum. After adding colistin sulphate (CS) and azidothymidine (AZT) alone or in combination according to the minimum plasma concentrations after 3rd dose (1h after IV infusion) bacteria were incubated at 37°C and 180rpm. Ctrl – control without any addition

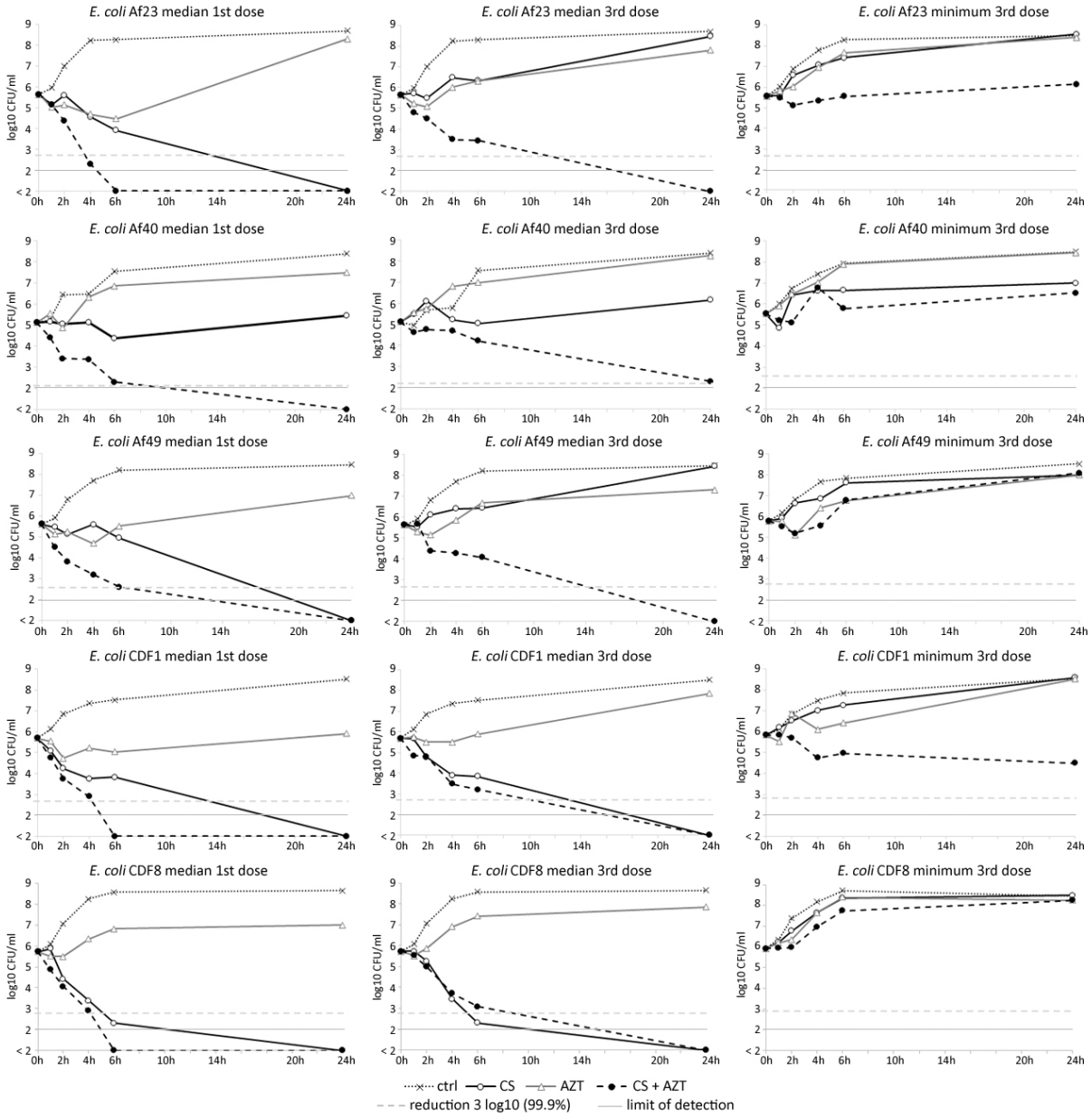

Figure 4 Time-kill curves in heat-inactivated serum of colistin-resistant strains

Bacteria were inoculated in heat-inactivated serum. After adding colistin sulphate (CS) and azidothymidine (AZT) alone or in combination according to the median and minimum plasma concentrations after 1st and/or 3rd dose (1h after IV infusion) bacteria were incubated at 37°C and 180rpm. Ctrl – control without any addition

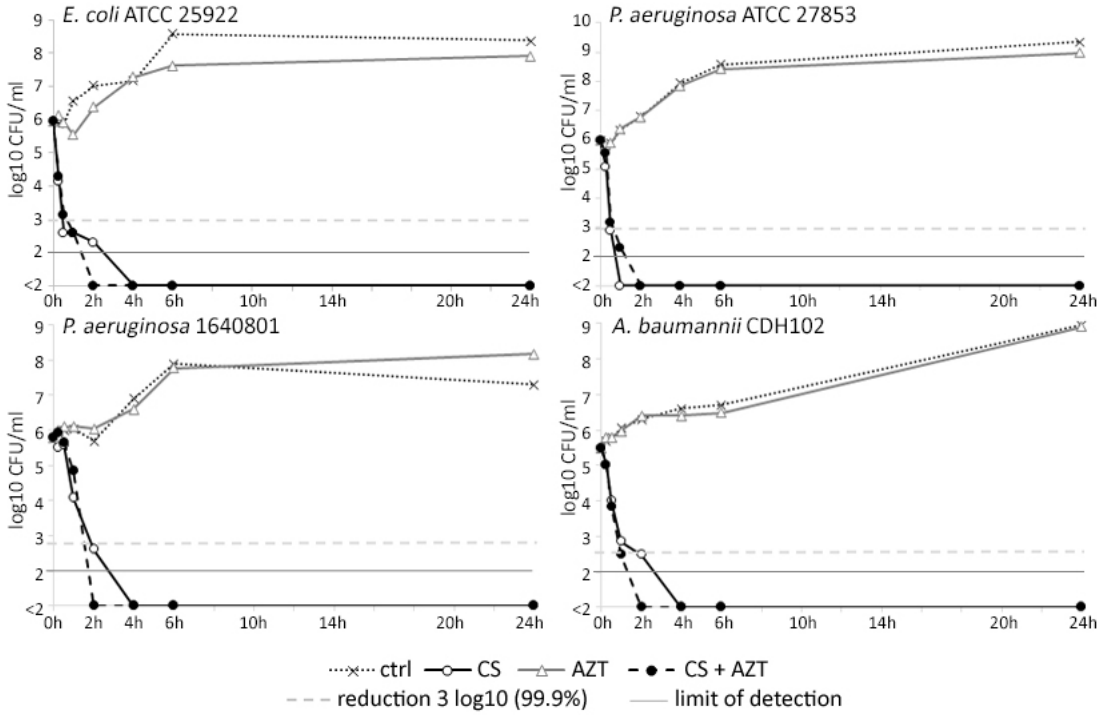

Figure 5 Time-kill curves in heat-inactivated serum of colistin-susceptible strains with minimum 3rd dose concentrations

Bacteria were inoculated in heat-inactivated serum. After adding colistin sulphate (CS) and azidothymidine (AZT) alone or in combination according to the minimum plasma concentrations after 3rd dose (1h after IV infusion) bacteria were incubated at 37°C and 180rpm. Ctrl – control without any addition

TABLE 1 Plasma concentrations of colistin and AZT obtained 1 h after dosage

	Colistin [mg/l]		AZT [mg/l]
	CB	≙CS	
	1st dose		
minimum	2.83	3.42	1.42
median	4.06	4.91	1.84
maximum	5.19	6.28	2.87
	2nd dose		
minimum	2.03	2.46	0.44
median	2.64	3.19	0.65
maximum	2.98	3.61	0.81
	3rd dose		
minimum	1.27	1.54	0.38
median	2.62	3.17	0.68
maximum	3.19	3.86	1.08

TABLE 2 Features of the *mcr-1* positive *E. coli* isolates

Strain	*mcr-1* positive plasmid type/ plasmid size (ca. kb)	Sequence type	Source	Co-Resistances	Co-resistance gene	Country of Isolation	Reference
E. coli Af23	IncI2/ 70	10	Human blood	AMX-CIP-SXT-TET	bla_{TEM-1}	ZA	[14]
E. coli Af40	IncI2/ ND	57	Human wound	AMX-CTX-CIP-SXT-TET-CHL-FOS	$bla_{CTX-M-55}$ + *fosA3* + *tetR*	ZA	[14]
E. coli Af49	ND/ ND	226	Human urine	AMX-CTX-CIP-SXT-TET-CHL-FOS	$bla_{CTX-M-55}$ + *fosA3* + *tetR*	ZA	[14]
E. coli CDF1	IncFIB/ 90	3077	Human blood	AMX-SXT-TET-CIP-GEN	bla_{TEM-1}	CH	-

E. coli CDF8	Incx4/ 30	167	Human blood	AMX-CTX-CIP	bla_{CTX-M}	CD	-

AMX – Amoxicillin; CHL – Chloramphenicol; CIP – Ciprofloxacin; CTX – Cefotaxime; FOS – Fosfomycin; GEN – Gentamicin; KAN – Kanamycin; SXT – Trimethoprim/Sulfamethoxazole; TET – Tetracycline, ZA – South Africa; CH – Switzerland; ND – not determined

TABLE 3 Median (range) MIC values of CS in CAMHB and MBC values of CS and AZT in CAMHB, native and heat-inactivated serum

	CAMHB CS MIC/MBC		Serum MBC		Heat-inactivated serum MBC	
	MIC CS (mg/l)	MBC CS (mg/l)	CS (mg/l)	AZT (mg/l)	CS (mg/l)	AZT (mg/l)
E. coli Af23	8 (4-8)	8 (4-32)	0.25 (0.06-0.25)	0.5 (0.25-1)	4 (4-8)	32 (16->32)
E. coli Af40	8 (4-8)	16 (16-32)	0.50 (0.13-1)	>16 (16->16)	4 (4-8)	>32 (>32)
E. coli Af49	8 (8-16)	16 (8-32)	8 (2-8)	>16 (16->16)	8 (8)	>32 (>32)
E. coli CDF1	4 (4-16)	8 (4-16)	0.5 (0.5-1)	16 (8->16)	8 (4-8)	32 (16-32)
E. coli CDF8	8 (4-16)	16 (4-16)	2 (0.06-2)	8 (1-16)	8 (8-16)	>32 (32->32)
E. coli ATCC25922	1 (0.25-1)	1 (0.25-1)	0.13 (0.06-0.13)	0.25 (0.25-1)	2 (1-2)	32 (32->32)
P. aeruginosa ATCC27853	1 (0.5-2)	1 (0.5-2)	0.5 (0.5-2)	>16 (>16)	4 (4-8)	>32 (>32)
P. aeruginosa 1640801	0.375 (0.25-1)	2 (1-4)	2 (1-8)	>16 (>16)	2 (2-4)	>32 (>32)
A. baumannii CDH102	1 (0.5-1)	1 (1)	2 (0.5-2)	>16 (>16)	1 (1-2)	>32 (>32)