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ARTICLE

Target site antimicrobial activity of colistin might be misestimated if tested in conventional growth media

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Abstract Cation-dependent inhibition of antimicrobial activity was reported for polymyxin antibiotics. Ca^{2+} and Mg^{2+} concentrations recommended by the Clinical and Laboratory Standards Institute (CLSI) for the supplementation of Müller–Hinton broth (MHB) are markedly lower than interstitial space fluid (ISF) concentrations in vivo. Hence, it was speculated that the antimicrobial activity of colistin might be overestimated if tested using conventional cation-adjusted MHB. The antimicrobial activity of colistin against $n=100$ clinical isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* ($n=25$ each) was evaluated by broth microdilution and, for selected isolates, by time–kill curves, in MHB without cations (MHB_{ONLY}), MHB supplemented with 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+} according to CLSI recommendations (MHB_{CLSI}), and in MHB adjusted to 50 mg/L Ca^{2+} and 20 mg/L Mg^{2+} simulating ISF concentrations (MHB_{ISF}). The minimum inhibitory concentration (MIC) values of colistin against the vast majority of isolates of both *P. aeruginosa* and *A. baumannii* increased significantly with higher cation concentrations. The susceptibility of *K. pneumoniae* isolates to colistin did not show significant changes between cation-supplemented media, while the MICs of *E. coli* decreased with ascending cation concentrations. These findings were confirmed in time–kill studies, where colistin killing against *P. aeruginosa* 1514 and *A. baumannii* 1485 declined with increasing cation concentrations. Contrarily, the killing of selected concentrations of colistin against *K. pneumoniae* 15 and *E. coli* 16 was enhanced in the presence of increasing cation concentrations. The present data suggest that the clinical antimicrobial activity of colistin might be misestimated in vitro if tested in conventional growth media.

Introduction

Colistin (polymyxin E) is a polypeptide antibiotic belonging to the antibiotic class of the polymyxins. In recent years, the clinical use of colistin has greatly increased due to its activity against a variety of multidrug-resistant Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella* spp. In analogy to polymyxin B, colistin is believed to interact electrostatically with the outer membrane of Gram-negative bacteria and to competitively displace divalent cations (calcium and magnesium) from the negatively charged phosphate groups of membrane lipids [1], thereby disrupting the integrity of both the outer and the cytoplasmic membrane, and ultimately leading to cell death.

Not surprisingly, increased concentrations of both calcium (Ca^{++}) and magnesium (Mg^{++}) were already shown to hamper or completely abolish the antibacterial efficacy of polymyxins [2–4]. Clinical and Laboratory Standards Institute (CLSI)-recommended cation concentrations for cation-adjusted Müller–Hinton broth (CA-MHB), the standard bacterial growth medium for microbiological susceptibility testing, range from 20 to 25 mg/L for Ca^{++} and from 10 to 12.5 mg/L for Mg^{++} [5]. However, reference ranges for ionised extracellular Ca^{++} and Mg^{++} concentrations are 46–53 mg/L and 16–26 mg/L, respectively [6]. Thus, while Mg^{++} concentrations in CA-MHB appear to be quite close to those seen in vivo, disparity between Ca^{++} concentrations in human interstitial space fluid

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(ISF) and those in CA-MHB is more evident, with the concentrations in vivo being about twice as high.

In light of these considerations, it seems possible that microbiological tests involving polymyxin antibiotics performed with conventional CA-MHB might overestimate the antimicrobial activity of the tested compounds. However, most of the available data in this context are in regards to polymyxin B. Therefore, the present study set out to investigate the influence of different cation concentrations on the antimicrobial activity of colistin, by both broth microdilution and time–kill studies.

Materials and methods

Colistin sulphate (further referred to as 'colistin') was purchased from Sigma-Aldrich, Vienna, Austria. A total of 100 clinical isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* (n= 25 for each bacterial species) were kindly provided by the Clinical Division of Clinical Microbiology, Clinical Institute of Laboratory Medicine, Medical University of Vienna.

Growth media

MHB without cations was purchased from Sigma-Aldrich, Vienna, Austria, and was used as a basis for the preparation of bacterial growth media. All experiments were conducted in three sets of growth media containing different calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations. One stock of pure MHB was left void of cations and will be further referred to as MHB_{ONLY} in the manuscript. For the preparation of cation-adjusted MHB according to CLSI standards (further referred to as MHB_{CLSI}), $CaCl_2$ and $MgCl_2$ solutions were added to give final Ca^{2+} and Mg^{2+} concentrations of 25 and 12.5 mg/L, respectively. For the preparation of MHB meant to simulate ISF cation concentrations (further referred to as MHB_{ISF}), Ca^{2+} and Mg^{2+} levels were adjusted to 50 and 20 mg/L, respectively.

Broth microdilution

The minimal inhibitory concentrations (MICs) of colistin against 100 clinical isolates of *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli* (n=25 for each species) were determined in non-treated Nunc™ 96-well round bottom polystyrene microwell plates by a broth microdilution method in accordance with CLSI recommendations. Experiments were conducted in duplicate in: (I) MHB_{ONLY}, (ii) MHB_{CLSI} and (iii) MHB_{ISF}, as specified above.

Time–kill studies

The antibacterial activity of different concentrations of colistin against selected isolates which showed the most prominent increase in MIC from MHB_{CLSI} to MHB_{ISF} was investigated in time–kill studies. As in microdilution, the employed media were: (I) MHB_{ONLY}, (ii) MHB_{CLSI} and (iii) MHB_{ISF}. Colistin concentrations were chosen to cover the full MIC range observed in microdilution testing for the respective organism. Experiments were performed in triplicate.

Results

Microdilution testing

The mean±standard deviation (SD) colistin MIC values against the tested strains, including ratios between MICs in the respective media, are summarised in Table 1. The activity of colistin against *P. aeruginosa* isolates clearly decreased in the presence of ascending cation concentrations. The mean colistin MIC values increased significantly from 1.50± 0.71 mg/L in MHB_{CLSI} to 2.72±0.98 mg/L in MHB_{ISF} (p= <0.001), reflecting cation-dependent MIC increases of at least one dilution step in the vast majority of isolates. For two isolates, the MICs were identical between MHB_{CLSI} and MHB_{ISF}, and only in two cases was the colistin activity higher in MHB_{ISF} than in MHB_{CLSI}. The colistin activity in MHB_{ONLY} (mean MIC=0.49±0.42 mg/L) was superior to the two cation-supplemented test media in all but two cases.

A similar cation-dependent inhibition of the colistin activity was observed in the assays with *A. baumannii*. Against the majority of tested isolates, colistin MICs rose by at least one dilution step with increasing cation concentrations, leading to mean MIC values of 1.19±0.74 mg/L in MHB_{CLSI} and 2.52± 3.05 mg/L in MHB_{ISF}. In analogy to *P. aeruginosa*, the difference in MICs between MHB_{CLSI} and MHB_{ISF} was statistically significant (p=<0.001). In four cases, the MIC did not change between the two cation-supplemented media, and two isolates displayed higher MICs in MHB_{CLSI} than in MHB_{ISF}. Again, the colistin activity was constantly higher in MHB_{ONLY} (mean MIC=0.39±0.23 mg/L) than in MHB_{CLSI} and MHB_{ISF}.

The colistin activity against *K. pneumoniae* was highly variable between the tested isolates and seemed virtually unaffected by differences in cation concentrations. The mean colistin MIC values did not change significantly between the two cation-supplemented growth media (p=0.076).

A different pattern emerged for *E. coli*. Here, in contrast to the results observed for *P. aeruginosa* and *A. baumannii*, the colistin activity showed a linear increase with ascending cation concentrations. Accordingly, the mean MIC values were highest in MHB_{ONLY} and lowest in MHB_{ISF}, with a

Table 1 Minimal inhibitory concentrations (MICs, in mg/L) of colistin against clinical isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* (n=25 each) in: (a) MHB_{ONLY}, (b) MHB_{CLSI} and ©) MHB_{ISF}. (d) Ratios of colistin MIC in MHB_{CLSI} versus MHB_{ONLY}. (e) Ratios of colistin MIC in MHB_{ISF} versus MHB_{ONLY}. (f) ratios of colistin MIC in MHB_{ISF} versus MHB_{CLSI}

	P.aeruginosa	*A.baumannii*	*K.pneumoniae*	*E. coli*
(a) MIC (mg/L) in MHB_{ONLY}	0.49±0.42	0.39±0.23	1.71±3.13	0.71±0.77
(b) MIC (mg/L) in MHB_{CLSI}	1.50±0.71[a]	1.19±0.74[a]	1.60±6.47[a]	0.43±0.29
©) MIC (mg/L) in MHB_{ISF}	2.72±0.98[a,b]	2.53±3.05[a,b]	1.68±6.46[a]	0.33±0.11[a,b]
(d) MHB_{CLSI}/MHB_{ONLY}	4.79±3.31	3.74±1.96	0.45±0.45	1.06±0.80
(e) MHB_{ISF}/MHB_{ONLY}	9.30±6.49	7.24±6.28	0.49±0.46	0.95±0.65
(f) MHB_{ISF}/MHB_{CLSI}	2.05±0.85	1.97±1.13	1.33±0.81	1.02±0.66

Values are displayed as mean±standard deviation (SD)

[a] Statistically significant difference to MHB_{ONLY}

[b] Statistically significant difference to MHB_{CLSI}

statistically significant drop in the MIC from MHB_{CLSI} and MHB_{ISF} (p=0.039).

Time–kill studies

P. aeruginosa isolate 1514, *A. baumannii* isolate 1485, *K. pneumoniae* isolate 15 and *E. coli* isolate 16 were chosen to test the killing activity of colistin over time. The MIC values of the selected bacteria in different test media and colistin concentrations evaluated in time–kill experiments against the respective organisms are shown in Table 2. Against *P. aeruginosa* 1514, colistin showed a distinct pattern of reduced bacterial killing after 5 h of exposure to increasing cation concentrations (Fig. 1a). This effect was most prominent at the highest concentration tested (8 mg/L), where colistin killing was reduced by 3 log steps in MHB_{ISF} compared to MHB_{CLSI}. This difference in activity could be observed to a lesser degree also at 4 mg/L, where colistin killing was reduced by approximately 1 log step in MHB_{ISF}. At 1 mg/L, colistin had virtually no activity in MHB_{ISF}, while it retained at least some effect in MHB_{CLSI}, allowing only a slight growth. At the lowest concentration tested, colistin was ineffective against *P. aeruginosa* 1514 in both cation-supplemented media (not shown).

Table 2 Colistin MIC values determined by broth microdilution in different media and concentration ranges chosen for the time–kill studies for *P. aeruginosa* 1514 (PA1514), *A. baumannii* 1485 (AB1485), *K. pneumoniae* 15 (KP15) and *E. coli* 16 (EC16)

	Mean colistin MICs in broth microdilution (mg/L)			Concentrations tested in time–kill studies (mg/L)
	MHB_{ONLY}	MHB_{CLSI}	MHB_{ISF}	
PA1514	0.375	1	4	0.375, 1, 4, 8
AB1485	0.1875	1	3	0.2, 1, 3, 6
KP15	1	0.25	0.75	0.25, 0.5, 2, 8
EC16	0.125	0.125	0.3125	0.25, 0.5, 1, 4

A similar pattern emerged for *A. baumannii* 1485 (Fig. 1b). Here, however, divergence in killing was highest at colistin concentrations of 3 and 1 mg/L. In both cases, the reduction in colistin killing after 5 h of incubation in MHB_{ISF} compared to MHB_{CLSI} amounted to approximately 3 log steps. At 3 mg/L, colistin attained a colony count reduction of almost 4 log steps in MHB_{CLSI} compared to 1 log step in MHB_{ISF}. At 1 mg/L, colistin was still bactericidal in MHB_{CLSI}, while in MHB_{ISF}, it hardly achieved growth inhibition. At 0.2 mg/L, colistin was ineffective against the tested organism in all three media (not shown). Against both *P. aeruginosa* 1514 and *A. baumannii* 1485, colistin performed best in MHB_{ONLY}, with killing activity equal or superior to that seen in the two cation-supplemented media.

Contrarily, the killing of colistin against *K. pneumoniae* 15 seemed to improve with increasing cation concentrations (Fig. 2a). At the highest tested concentration (8 mg/L), colistin was rapidly bactericidal in MHB_{ISF}, achieving complete and sustained killing already at 2 h. In comparison hereto, at 5 h, killing was reduced by 3 and 6 log steps in MHB_{CLSI} and MHB_{ONLY}, respectively. However, this discrepancy between the different media was less evident at 2 mg/L of colistin, where reduction in colony counts was only slightly superior in MHB_{ISF} compared to MHB_{CLSI} and MHB_{ONLY}. At the two lowest concentrations, colistin was ineffective against *K. pneumoniae* 15 in all growth media, with colony counts identical to growth control.

Against *E. coli* 16, 4 mg/L of colistin were also rapidly bactericidal in MHB_{ISF}, with sterile conditions achieved at 5 h, while killing was reduced by 3 and 6 log steps in MHB_{CLSI} and MHB_{ONLY}, respectively (Fig. 2b). Colistin concentrations of 1 mg/L still achieved a more than 3 log step colony count reduction in MHB_{ISF} and MHB_{CLSI}, while they

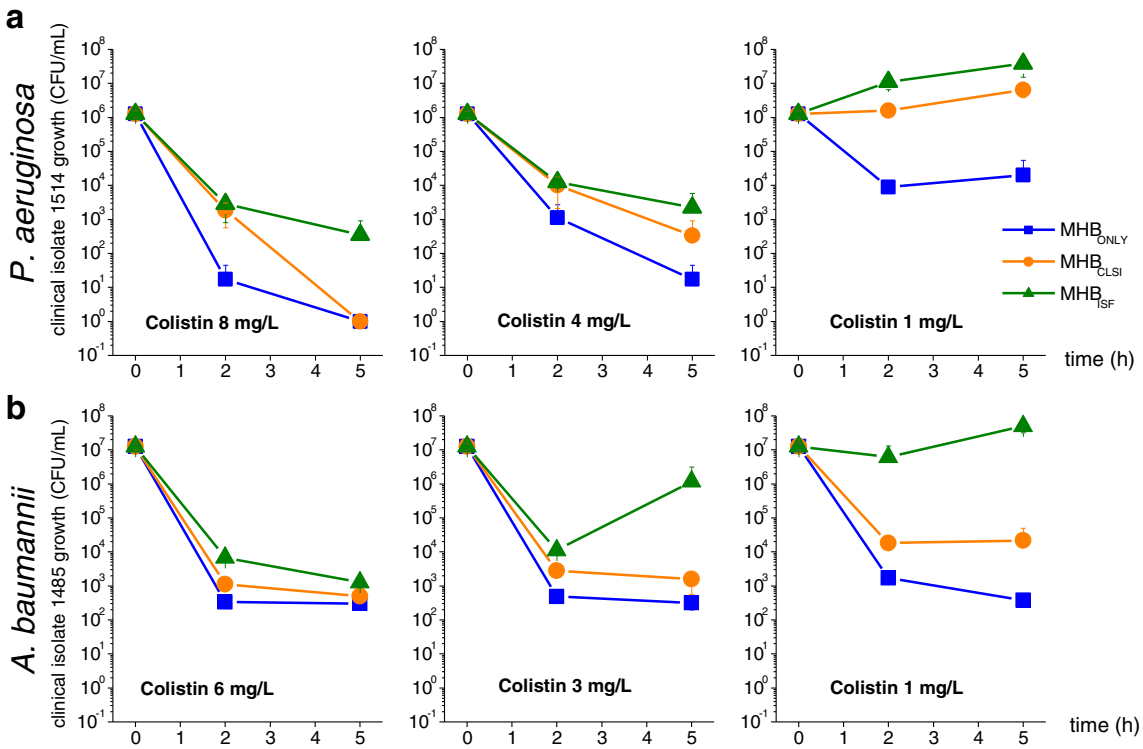

Fig. 1 Time–kill studies of different concentrations of colistin against: (**a**) *Pseudomonas aeruginosa* clinical isolate 1514 and (**b**) *Acinetobacter baumannii* clinical isolate 1485 in MHB_{ONLY} (*squares*), MHB_{CLSI} (*circles*) and MHB_{ISF} (*triangles*), respectively

were ineffective in MHB_{ONLY}. Lower colistin concentrations did not show any activity against *E. coli* 16 in any of the employed media.

Throughout all experiments, the growth curves of all investigated organisms were not affected by differences in cation concentrations in the employed test media.

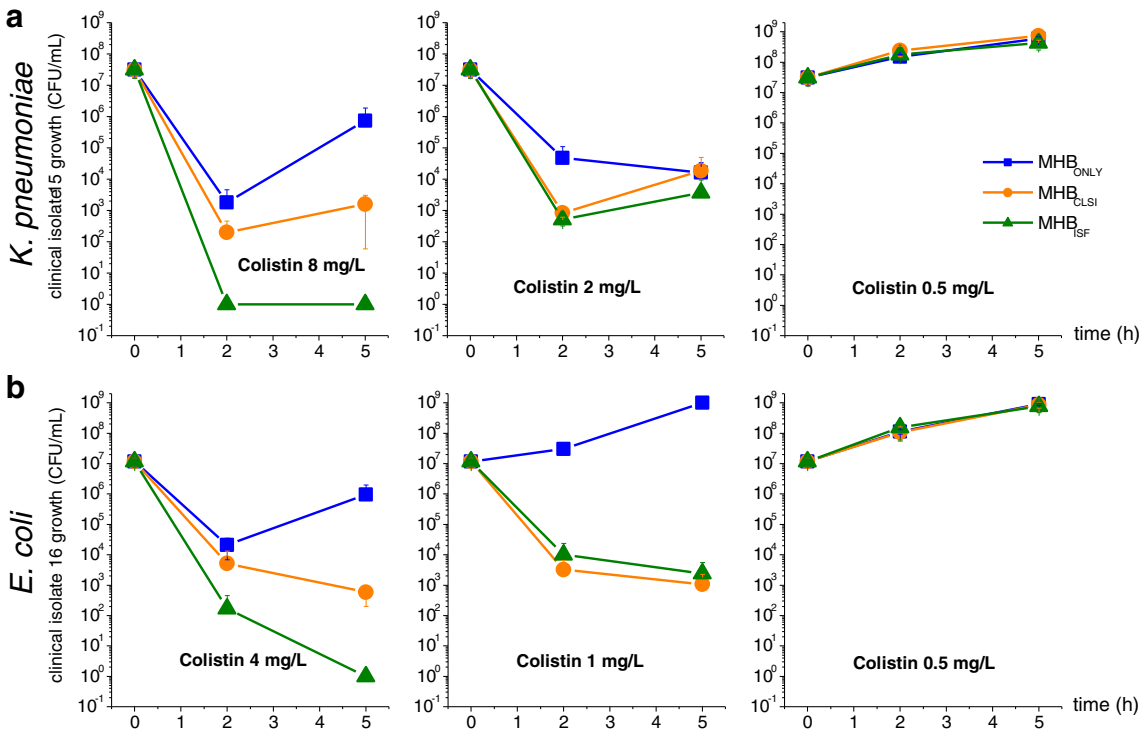

Fig. 2 Time–kill studies of different concentrations of colistin against: (**a**) *Klebsiella pneumoniae* clinical isolate 15 and (**b**) *Escherichia coli* clinical isolate 16 in MHB_{ONLY} (*squares*), MHB_{CLSI} (*circles*) and MHB_{ISF} (*triangles*), respectively

Discussion

The present study investigated the influence of different cation concentrations on the antibacterial activity of colistin as determined by broth microdilution and time–kill studies. Colistin activity was observed to decline markedly in the presence of higher cation concentrations when tested against the vast majority of *P. aeruginosa* and *A. baumannii* isolates, whereas, on the contrary, increasing cation concentrations partly enhanced colistin's activity against *K. pneumoniae* and *E. coli*.

In general, in vitro susceptibility tests are performed in order to estimate the ability of an antimicrobial drug to treat infections caused by certain pathogens in vivo. The data presented here demonstrate that, due to cation-dependent changes in the antimicrobial activity of colistin for tissue, these estimates might not hold true, depending on the bacterial species.

The results observed for *P. aeruginosa* and *A. baumannii* indicate that colistin's antibacterial activity against these two species in vivo might be overestimated by using conventional in vitro growth media. Importantly, it must be mentioned that colistin MIC values against *P. aeruginosa* did not exceed the current breakpoint of 4 mg/L, even for those isolates with the strongest cation-dependent loss in susceptibility. In contrast, for *A. baumannii*, in some cases, changes in the MIC between MHB_{CLSI} and MHB_{ISF} did determine a breakpoint shift from susceptible to resistant, i.e. the MIC values of five *A. baumannii* isolates were above the currently established breakpoint of 2 mg/L when tested in MHB_{ISF} but would have been classified as susceptible in MHB_{CLSI}. This might be of clinical relevance since the selection of colistin to treat a pathogen only allegedly susceptible in vitro, but resistant in vivo, could lead to treatment failure and promote resistance. Based on the present data, supplementation of employed growth media with cation concentrations adjusted to values of human ISF would seem reasonable at least for *P. aeruginosa* and *A. baumannii*.

Data derived from investigations with *K. pneumoniae* and *E. coli* point to a different direction. In time–kill studies against both species, selected concentrations of colistin showed a linear augmentation of antibacterial killing in the presence of ascending cation concentrations. While for *E. coli* microdilution experiments had already anticipated a pattern of cation-dependent increase in activity, colistin MIC values for *K. pneumoniae* were apparently unaffected by different cation concentrations. However, the mentioned variability of MIC values between *K. pneumoniae* isolates might have masked a de facto existing trend. These results, in overt contrast to those observed for *P. aeruginosa* and *A. baumannii*, are quite unexpected. While a cation-dependent inhibition of colistin's activity correlates well with the current understanding of the drug's mechanism of action, the cation-dependent augmentation of activity observed against *K. pneumoniae* and *E. coli* seems to be lacking an underlying explanation, and highlights the need for further investigations. However, the strikingly discrepant results observed for *P. aeruginosa* and *A. baumannii* on the one hand and *K. pneumoniae* and *E. coli* on the other hand suggest that the influence of divalent cations on the antimicrobial activity of colistin might be species-dependent. If confirmed, these findings might also require additional precautions in association with the antimicrobial susceptibility testing of colistin.

The need for a revision and adaptation of the current recommendations regarding antimicrobial susceptibility testing of colistin has been highlighted previously. Colistin is a polycationic molecule known to adhere to inorganic materials, including labware, which might result in considerable loss of the compound during experimental conditions [7]. In accordance with this hypothesis, the results of colistin broth microdilution experiments have been shown to differ significantly if conducted in microtitre plates with differently coated wells [8]. In analogy, colistin MICs against relevant pathogens have been observed to decline in the presence of Polysorbate 80, a surfactant often used as a dispersing agent in broth microdilution panels [8, 9]. Finally, comparison of the broth microdilution method with the Etest and the agar dilution method against selected multidrug-resistant Gram-negative organisms showed significant variability between the observed MIC results [10]. In light of the fact that the broth microdilution assay is currently the only method recommended by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for colistin antimicrobial susceptibility testing, incorporation of the above-mentioned factors in a revised edition of methodological recommendations for colistin antimicrobial susceptibility testing should be encouraged.

As reflected by the large SDs, some of the data presented here are affected by high variability. Also, even though a considerable number of clinical isolates was included, these experiments cannot claim to be representative for the totality of the populations of the respective bacterial strains. Moreover, it might be questioned as to what extent experiments in an artificial growth medium like MHB are representative for conditions in the human interstitial space in vivo. Nevertheless, these experiments have shown that the antibacterial activity of colistin is cation-dependent, and might be over- or underestimated in a species-dependent manner if tested in conventional, cation-poor growth media. Together with other recent reports, the findings of this study indicate the need for the adaptation of methodological recommendations regarding colistin antimicrobial susceptibility testing.

Conflict of interest Peter Matzneller declares that he has no conflict of interest.
Sabine Strommer declares that she has no conflict of interest.
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Markus Zeitlinger declares that he has no conflict of interest.

Compliance with ethical requirements This article does not contain any studies with human or animal subjects.

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