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Clinical validation of the SensiTest™ Colistin, a broth microdilution based method to evaluate colistin MICs

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RUNNING TITLE: validation of the SensiTest™ Colistin

ABSTRACT

The global spread of multi-drug resistant Gram negative has led to the return of colistin for treating severe infections. Recently, different plasmid-mediated genes conferring resistance to this drug were described and reported worldwide. International committees (EUCAST/CLSI) re-evaluated inconsistencies surrounding colistin antimicrobial susceptibility testing (AST), concluding that broth microdilution (BMD) should serve as the reference method for AST. The development of an accurate, reproducible commercial test based on BMD is therefore highly desirable. The SensiTest™ Colistin (STC), a BMD-based compact 4-test panel, containing the lyophilized antibiotic in 7 two-fold dilutions (0,25 - 16 µg/ml) was here compared with the EUCAST-CLSI standard reference method (BMD), and for some isolates, with the automated system Phoenix 100™ (PHX). A total of 353 bacterial strains were evaluated by two different laboratories; 137 isolates were resistant to colistin (19 intrinsically, 83 harboring the *mcr*-1 gene). Essential agreement (EA) between STC and BMD was obtained for 339 out of the 353 strains tested (96,0%). The overall categorical agreement was obtained for 349 out of the 353 strains analyzed (98,9%). Two major errors (MEs, 0,93%) and two very major errors (VMEs, 1,46%) were documented. STC appeared as a simple but highly reliable test, with a good reproducibility even with panels stored at room temperature or at 35°C. Moreover, STC showed a good performance on strains carrying the *mcr*-1 gene, with a 98,8% EA. As secondary endpoint of our study, VMEs for PHX were documented for 6 isolates (10%).

KEYWORDS: multi-drug resistance, colistin, MIC, SensiTest™ Colistin, antimicrobial susceptibility testing, Phoenix-100™, Liofilchem, Becton Dickinson.

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INTRODUCTION

The antibiotic properties of polymyxins, originally derived from strains of *Paenibacillus* (*Bacillus*) *polymyxa,* were first described in the 1940s. Formerly studied in the 1950s (1) and used for some years in the treatment of Gram-negative infections, colistin (polymyxin E) is the drug most widely used in clinical practice. However, with growing concern over significant side effects, colistin lost importance in comparison to emerging more efficacious drugs with less overt toxicity (2) and was removed from use some decades later.

The global spread of multi-drug resistant Gram negative bacteria (MDRGNB) has led to a distinct limitation in the therapeutic options available. This has seen the return of colistin to the clinical arena (3), albeit reassessed to better define its dosage and daily administration (4). Colistin has often become the last option to treat severe infections caused by MDRGNB, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and carbapenem-resistant *Enterobacteriaceae* (CRE), being frequently used in a dual therapy regimen.

Until recently, colistin resistance was always thought to be chromosomally encoded and mutationally acquired allowing vertical transmission only, and thus, by its very nature, rare and self-limiting. This kind of colistin resistance is determined by mutations in a wide variety of species-specific mechanisms; *mgrB* and *ccrB* in *Klebsiella pneumoniae*, *ParS* and *CprS* in *P. aeruginosa*, in addition to the more widely dispersed two component systems *PhoP-PhoQ* and *PmrA-PmrB* (2, 5-8). Universally however, these mechanisms involve the reduction of LPS net-charges, compromising the binding of cationic polymyxins.

Recently mobile colistin resistance, in the form of the plasmid-mediated *mcr*-1 gene was described: this gene encodes for a phosphoethanolamine transferase (9). Following its initial discovery, *mcr*-1 has been reported worldwide (10). At least twelve variants of this gene, which

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encode phosphoethanolamine transferase enzymes that differ from *mcr*-1 at a single amino acid, have been described. Immediately after the discovery of this first gene, other mobile elements significantly different from *mcr-1* have been described: *mcr*-2 was found in Belgium and *mcr*-3 was identified in Malaysia, Thailand and from the United States (11, 12). Most recently, *mcr*-4 was characterized in an Italian strain of *Salmonella enterica* serovar Typhimurium that was originally isolated in swine in 2013. This gene was also demonstrated to have had a circulation in the veterinary environment in Belgium and Spain, in strains collected in 2015 and 2016 (13). *Mcr*-5 was instead described in Germany in an isolate of *Salmonella enterica* serovar Paratyphi B, allowing postulation that the transfer of resistance genes from bacterial chromosomes to mobile genetic elements has occurred in multiple independent events (14).

Almost contemporarily with the emergence and dissemination of *mcr*-1, international committees (EUCAST/CLSI joint working group) sought to address the inconsistencies surrounding antimicrobial susceptibility testing (AST) of colistin. They concluded that broth microdilution (BMD) should serve as the reference method for testing colistin/polymyxin compounds. Owing to the large size and cationic nature of polymyxins, disk diffusion and gradient diffusion have been demonstrated to be unreliable (15). Additionally, agar dilution is not logistically feasible in clinical settings. Therefore, it follows that the development of commercial test for colistin AST based on BMD is highly desirable.

Here we present the clinical evaluation of the SensiTest™ Colistin (STC, Liofilchem, Italy), a compact 4-test panel containing the lyophilized antibiotic in 7 two-fold dilutions (0.25-16 µg/ml) with one additional well as growth control. The system will be proposed to evaluate colistin AST using a BMD method that complies the recommendations of international standards (i.e. CLSI, EUCAST) in a simpler and less time-consuming way.

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STC was compared with the classical BMD technique, performed according to the recommendations of CLSI and EUCAST and used as gold standard, and, for some isolates, also with the results of an automated system, the Phoenix 100™ (PHX, Becton Dickinson, USA).

RESULTS

The study results are summarized in table 1. All the tests performed were considered valid (presence of growth in the growth-control well). Only in a few cases was the evaluation of growth challenging, due to the presence of pinpoint colonies scattered into the broth. That occurred mainly for *Hafnia alvei* strains. Examples of the STC panels are shown in Figure 1.

Agreement between STC and BMD – Essential agreement (EA) was obtained for 339 out of the 353 strains tested (96.0%). In particular, for 151 strains the same MIC value was obtained for the two methods, whereas a difference ± 1 dilution was documented for 188 isolates.

Discrepancies of 2 or more dilutions were documented for 14 strains, 6 from the Italian clinical collection, 7 among the strains collected in Egypt and for one *mcr*-1 isolate collected in Thailand. The isolates were 6 *Escherichia coli*, 4 *K. pneumoniae*, 2 *Enterobacter aerogenes*, one strain of *H. alvei* and one isolate of *Salmonella* species. The discrepancy did not change the strains' clinical categorization in twelve of these cases; the other two cases were one isolate of *E. aerogenes* (BMD = 0,25 mcg/ml and STC = 4 mcg/ml) and one *Salmonella* species (BMD = 4 mcg/ml and STC = 1 mcg/ml). The overall categorical agreement (CA) was obtained for 349 out of the 353 strains analyzed (98.9%). Two MEs were recognized for one strain of *E. aerogenes* (as above) and one strain of *K. pneumoniae* (a *mcr*-1 positive isolate with BMD = 2 mcg/ml and STC = 4 mcg/ml), whereas two VMEs were documented for one strain each of *Salmonella* species (as above) and *E.*

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coli (BMD = 4 mcg/ml and STC = 2 mcg/ml). The rate of MEs for STC was 0,92% (2 isolates out of the 216 susceptible strains), whereas the rate of VMEs was 1,46% (2 out of 137 isolates).

Reproducibility of the STC – The six strains gave a total of 60 replicates. A total agreement among replicates (i.e., same MIC) was documented in 50 out of the 60 tests performed (83.3%), whereas the EA was 100%.

Stability of the STC – Stability tests on the STC panels were performed across different time periods with the STC panels stored at different temperatures, as shown in Table 2. All the tests fell into the agreement within ±1 two-fold dilution. Only one was out of range: the MIC at T7 of the reference strain NCTC-13846 (*E. coli* harboring the *mcr*-1 gene) for the panels stored at 4°C, which was 2 dilutions higher than expected.

The STC appeared also stable if MICs are evaluated in different times for the different rows of the same panel (Table 3).

Agreement between BMD and automated systems – PHX showed an overall agreement with BMD for 212 out of the 219 isolates analyzed (96.8%). The 7 discordant strains resulted in one ME for a *P. aeruginosa* strain, and 6 VMEs (two *E. coli* – one harboring the *mcr*-1 gene, two *Salmonella* species, one *H. alvei* and one *K. pneumoniae*), accounting for the 2.7% of the isolates tested.

DISCUSSION

Colistin is often considered as the last resource for the treatment of severe Gram-negative infections, in particular those caused by *P. aeruginosa*, *A. baumannii* and CRE (3). This compound has no activity against Gram-positive strains and against some Gram-negative such as *Burkholderia*

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cepacia, *Elizabethkingia meningoseptica*, *H. alvei*, *Morganella morganii*, *Proteus* species, *Providencia* species, *Serratia marcescens*, *Yersinia pseudotuberculosis* (2, 16, 17).

Immediately after the recent discovery of a plasmid-mediated mechanism of resistance that more often conveys a low-level resistance (MICs of 4-8 µg/ml) (9), strains harboring the gene have been described worldwide (mostly in *E. coli*) (10, 18). More recently, other mobile genetic resistance traits have been described (11-14). Thus, the spread of colistin-resistant microorganisms has become matter of concern worldwide, and the ECDC published in June 2016 a document calling for a rapid risk assessment in order to control the spread of plasmid-mediated colistin resistance in Enterobacteriaceae (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/enterobacteriacea e-risk-assessment-diseases-caused-by-antimicrobial-resistant-microorganisms-europe-june-2016.pdf). This document highlights different actions for the implementation of surveillance strategies and antimicrobial stewardship about this topic. Moreover, the development of improved laboratory methods for the determination of the correct colistin MIC and molecular *mcr*-1 detection are considered beneficial and necessary (19).

For colistin, a reliable evaluation of MICs, together with the possibility of therapeutic drug monitoring (TDM) is also necessary for the correct management of the patients, given colistin's potential significant adverse effects, in order to avoid toxic effects while maintaining sufficient antibacterial activity (19).

A joint EUCAST and CLSI subcommittee issued recommendations in July 2016 (subsequently confirmed in updates of August and November 2016 and June 2017) confirming that broth microdilution, using untreated polystyrene, is so far, the only valid method and that disk diffusion does not work because of the poor diffusion of the large colistin molecule (15, 20). In this

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document the adhesive properties related to the cationic nature of colistin were emphasized, suggesting that the BMD test should be performed by using cation-adjusted Mueller-Hinton Broth without additives (in particular, no polysorbate-80 or other surfactants), with trays of untreated polystyrene. Colistin may adhere to BMD plates; this issue should be taken into account when developing new devices. In cases of major discrepancies with the result expected, verification of the free concentrations of colistin in the test panels may be warranted. The trays used to produce the STC panels are made of plain, untreated polystyrene, compliant with EUCAST-CLSI guidelines. The results obtained in our various STC experiments were congruent with that of BMD, and so it was decided that evaluation of the free concentrations of colistin in these cases was unnecessary.

Moreover, issues about the correct MIC estimation with some automated instruments have been recently highlighted. EUCAST suggests to the users of semi-automated devices to apply rigorous QC, checking with the manufacturer whether or not they are confident that their method to evaluate colistin gives correct results (20). Regarding automated systems, the performances of Vitek-2™ (BioMérieux, Marcy l'Etoile, France) and MicroScan™ (Beckman Coulter, CA, USA) against CRE and *mcr*-1-positive isolates have been recently evaluated, resulting in a high rate of VMEs for Vitek-2™ (36%, 4% for Microscan™), whereas no ME were documented for Vitek-2™ and 15,8% of MEs was demonstrated for MicroScan™ (21). The high rate of VMEs with the VITEK®-2 AST-GN colistin (cs01n) compared to agar dilution (the reference method used for cs01n development) and compared to BMD was also demonstrated by an internal investigation performed by BioMérieux (communication to customers, BioMérieux).

These data express the pertinence of the development of new diagnostic tool for the clinical microbiology laboratories. Nevertheless, standard BMD methods are labor-intensive and does not fall in the routine practice of the majority of the clinical microbiology laboratories.

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The SensiTest™ Colistin appears a simple but highly reliable test to assess the colistin susceptibility. The preparation procedure is easy and fast and the results evaluation is simple, reflecting that of the BMD methodology. With this device, it is possible to test a single drug, and therefore it may be used as a second line assay for laboratories which use automated instruments in their daily practice without redundancies for other antibiotics. Even if the panels are customized for testing four separate isolates at distinct timepoints, the MIC given by the STC appeared stable.

In our assessment, the device appeared to be highly reproducible. We tested the reproducibility of the panels stored at different degrees of temperature, reflecting conditions the STC panels may experience in real life use in particularly challenging conditions, such as those that may occur in developing countries, with possible troubleshooting due to transports and storage.

As ancillary result of our study, we demonstrated an overall good agreement between BMD and Phoenix-100™, even if with the limitation that PHX was performed as standard of care few days before the execution of the BMD. Among the 219 isolates tested also with PHX, the VMEs involved two strain each for *E. coli* (one *mcr*-1) and *Salmonella* species, one strain of *H. alvei* and one strain of *K. pneumoniae*. The rate of VMEs for the PHX was, in our study, 10% (6 isolates out of the 60 colistin resistant strains analyzed with the automated instrument).

The STC was recently evaluated by the EUCAST committee in June 2017, together with other commercially available BMD techniques. In that study, the STC was tested on 75 strains: the EA appeared to be 88%, with 7 MEs and one VME (20). In our study, the test performance was better, with EA = 96.0%, 2 MEs and 2 VMEs. A direct comparison between these data and our study appears difficult with likely disparity between the EUCAST tested strains and our clinically representative, geographically varied test panel.

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199 In our experience, the STC showed a very good performance on strains carrying the *mcr*-1 gene,
200 with a 98.8% EA (Table 1). For some strains, such as *H. alvei*, it is important to carefully evaluate
201 any growth in the wells, as it may present as pinpoint microcolonies for this species (figure 1).
202 However, it is likely that the recent caveat that reclassify this microorganism as constitutively
203 resistant to colistin (17), may render in future the evaluation of colistin AST for these strains
204 redundant.

205 Another possible advantage of using the STC is that, being an open system, it is possible to
206 evaluate also the minimal bactericidal concentration after the reading, by spotting 1 to 10 µl onto
207 a Mueller Hinton agar plate (figure 2) considering the growth after 24h.

208 In the present study, a limited number of *A. baumannii* and *P. aeruginosa* were tested and further
209 evaluation on these isolates may be of value. However, even if further assessments may be
210 warranted, perhaps in-field/in-clinic studies, our preliminary findings suggest that the STC could be
211 proposed both as a first-line test for selected specimens or as a confirmatory test adjacent to
212 automated screening.

213

214 **MATERIALS AND METHODS**

215 **Strains collections –** Three different sets of isolates have been analyzed by two different
216 laboratories (Clinical Microbiology Laboratory, Reggio Emilia, Italy – center A – and Department of
217 Medical Microbiology and Infectious Disease, Cardiff, UK – center B). A total of 353 bacterial
218 strains were evaluated.

219 216 out of the 353 strains were collected in Italy and analyzed in center A. 159 isolates were
220 prospectively collected from blood culture samples and analyzed as fresh isolates. 57 strains
221 belonged to laboratory collections of peculiar clinical isolates, collected in the previous two years

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and stored at -80°C in microbeads (Pro-Lab Diagnostics, USA). They were selected on the basis of the following characteristics: 45 Enterobacteriaceae with reduced susceptibility to colistin (7 of them were *Escherichia coli* carrying the *mcr-1* gene, as demonstrated using an *in-house* PCR); 12 carbapenem resistant *K. pneumoniae*; 159 strains isolated from blood cultures.

59 out of the 353 strains were collected over the previous 3 years as part of a clinical study in Tanta Teaching Hospital, Egypt, and were stored in Cardiff Laboratory at -80°C in microbeads (Pro-Lab Diagnostics). Of these, 20 Enterobacteriaceae were colistin-resistant (according to EUCAST breakpoints) with an un-defined mechanism and 39 strains were colistin susceptible. These strains were evaluated in center B.

Finally, 75 strains were collected in Thailand within the last 12 months, also stored in Cardiff Laboratory again at -80°C using microbeads. All these strains harbored the *mcr*-1 gene, confirmed by PCR. Also these isolates were analyzed in center B.

Finally, three reference strains (*E. coli* ATCC® 25922, *P. aeruginosa* ATCC® 27853, *E. coli* NCTC® 13846) were included as controls, and tested to validate the different experimental sessions in both Laboratories. A detail of the isolates analyzed in the present study is shown in table 1.

STC: strains analysis – Briefly, a 0.5 McFarland suspension of the microorganism to be tested was prepared in a solution of 0.90% w/v of NaCl (saline) and then diluted 1:20 always in saline, obtaining the solution A. 0.4 ml of solution A was then added to the 3.6 ml tube of Mueller-Hinton broth II provided in the STC kit, obtaining the solution B. 100 µl of solution B was then dispensed into each well in a row. The STC panels were then incubated at 36 ± 2°C for 16-20 hours in ambient air. The results were read visually by naked eyes of two different operators, by using bright, indirect lighting against a dark background. The presence of growth in the growth-control well was considered first, allowing the test to be considered valid. For MIC determination, bacterial growth

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was considered as the presence of turbidity, or button at the bottom of the well, or pinpoint colonies in the broth. Different lots of the panels were tested during the study period.

STC: evaluation of reproducibility and stability – These tests were performed at the Clinical Microbiology Laboratory, Reggio Emilia, Italy. Six different strains were chosen: the three QC reference strains (included the NCTC® 13846 *E. coli mcr*-1 positive), 2 *E. coli* (one, named CSR-55, with MIC = 8 mcg/ml with undefined resistance mechanism and the other, named CSR-57, with MIC = 2 mcg/ml) and 1 carbapenem resistant *K. pneumoniae* strain with MIC >= 16 mcg/ml due to mechanisms different from mobile determinants. All the isolates were tested as replicates 10-times.

The stability of the product was assessed in two ways. Firstly, three different boxes containing 32 panels were stored at 4°C (as suggested by the manufacturer), at room temperature, and at 35°C. Six different strains as above were tested the day after the arrival of the panels (T0), one week after (T7), two weeks after (T14), one month after (T30) and three months after the delivery of the products (T90).

Each single panel allows testing of up to four strains; if less than 4 tests have been performed, the manufacturer provides film with which to seal the inoculated rows (in order to prevent any leakage of contaminated fluids) and to return the panel into its own desiccant envelop and into the fridge. The stability of the panels used at different times was established by analyzing the six different strains as above with six different panels at day 0 (T0) (inoculum in the first row), and then after 5 (T5), 13 (T13) and 19 days (T19), storing in the meantime the panels in fridge and then inoculating the second, third and fourth row, respectively.

The expected values of MICs were those established by EUCAST Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion (version 7.0, valid from 2017-01-01) for

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the control strains, or for the other strains, a ±1 dilution of the MIC obtained in five previously performed BMD experimental sessions.

Broth microdilution – BMD was performed according to the ISO standard method (20776-1), which has been demonstrated to work well for Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* species. Colistin sulfate salt was bought from Sigma-Aldrich (Merck KGaA, Germany) as lyophilized powder (100 mg - 15.000 U/mg), that was resuspended in distilled water. Vials with a final concentration of 1024 mcg/ml were stored at -80°C until the different test sessions were performed. The cation-adjusted Mueller-Hinton broth (CAMHB) used was the ready for use Mueller-Hinton II (Liofilchem, Italy). The trays bought from Nuova Aptaca, Italy, were made of plain polystyrene and not treated in any way before use. No additives (Tween-80 or other surfactants) have been added in any part of the testing process.

For any working session, accounting for 20 isolates, eleven working concentrations of colistin (ranging from 0.064 µg/ml to 64 µg/ml in 2-fold dilutions) were prepared in separate tubes containing CAMHB, according to the dilution scheme proposed by CLSI (22). 50 µl of each intermediate concentration were dispensed into the wells of the microwell plates. For each strain tested a positive growth control was included in the first well of the plate.

Quality control strains were tested in the first session; each further experimental session was validated by using one of these strains added on rotation.

Isolated bacterial colonies were selected from a 18-24h blood agar culture and transferred to a CAMHB tube. The broth was incubated overnight at 35-37°C and the turbidity adjusted to 0.5 McFarland standard and the suspension was then diluted in broth to obtain a final bacterial concentration of 5×10^5 colony forming units/ml.

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Finally, 50 µl of bacterial suspension were added to each well of the 96-well microplates, which were incubated in an ambient air incubator at 35 °C ± 2°C for 16-20 hours. The MICs were determined as the lowest concentration that completely inhibits bacterial growth in the wells.

Phoenix 100™ - The tests were performed according to the manufacturer's recommendations as a part of the standard of care for the 219 Italian clinical isolates. The card NMIC-417™ was used for the present study.

Discrepant results – Discrepant results that showed a disagreement between BMD and STC with more than a two-fold dilution were repeated with both methods in at least two other different experimental sessions. The results of each test that were confirmed in more than two experimental sessions were considered as confirmed and used for the final analysis.

Agreement between methods – Essential agreement (EA) is defined as the agreement within plus or minus one two-fold dilution of STC with the MIC obtained through the reference method determination (BMD). EA determination was evaluated exactly for all the values below 16 mcg/ml (values of ≥16 mcg/ml were considered in agreement). EA between PHX and BMD was not analyzed due to the narrow MIC range tested by the PHX card (only three dilutions, 1-4 mg/L).

For discrepant results, we evaluated the categorical agreement (CA), i.e., when the results did not change the categorization of the isolates (considered as susceptible or resistant) or the occurrence of major errors (ME – the BMD result is S and the STC or the PHX result is R) or very major errors (VME – the BMD result is R and the STC or the PHX result is S) (23).

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314 Conflict of interest: none to declare.

315

316 **FIGURES AND TABLES**

Strains	Total (colistin resistant)*	Same MIC	±1 Dilution	EA (%)	CA (nr - %)	ME (nr.)	VME (nr.)
Acinetobacter baumannii	6 (0)	1	5	100,0	6 - 100%	0	0
Acinetobacter species[#]	4 (1)	3	1	100,0	4 - 100%	0	0
Citrobacter koseri	1 (0)	0	1	100,0	1 - 100%	0	0
Enterobacter aerogenes	4 (0)	1	1	50,0	3 - 75,0%	1	0
Enterobacter cloacae complex	4 (2)	1	3	100,0	4 - 100%	0	0
Escherichia coli	205 (89)	80	119	97,1	204 - 99,5%	0	1
Hafnia alvei	6 (6)	3	2	83,3	6 - 100%	0	0
Klebsiella oxytoca	6 (0)	2	4	100,0	6 - 100%	0	0
Klebsiella pneumoniae	76 (23)	34	38	94,7	75 - 98,7%	1	0
Leclercia adecarboxylata	1 (0)	0	1	100,0	1 - 100%	0	0
Morganella morganii	2 (2)	2	0	100,0	2 - 100%	0	0
Proteus mirabilis	5 (5)	5	0	100,0	5 - 100%	0	0
Providencia species	2 (2)	2	0	100,0	2 - 100%	0	0
Pseudomonas aeruginosa	19 (0)	9	10	100,0	19 - 100%	0	0
Salmonella species	7 (2)	3	3	85,7	6 - 85,7%	0	1
Serratia marcescens	4 (4)	4	0	100,0	4 - 100%	0	0
Shigella species	1 (1)	1	0	100,0	1 - 100	0	0
TOTAL	**353**	**151**	**188**	**96,0**	**349 - 98,9%**	**2**	**2**
Colistin susceptible	216	81	124	94,9	214 - 99,1%	2	N/A
Colistin resistant (not *mcr*-1)	54	43	9	96,3	53 - 98,1%	N/A	1
Colistin resistant (*mcr*-1)	83	27	55	98,8	82 - 98,8%	N/A	1
TOTAL	**353**	**151**	**188**	**96**	**349 - 98,9%**	**2**	**2**

Table 1 - Study results. Legend: EA = essential agreement, CA= categorical agreement, ME = major errors; VME = very major errors; N/A = not applicable. # = included one strain each for *Acinetobacter ursingii, A. lwoffii, A. junii, A. nosocomialis.* * = colistin resistance was defined according to EUCAST breakpoints.

	MIC: expected values (range)	panels stored at 4°C				
		T0	T7	T14	T30	T90
ATCC-25922	0,25 – 2*	1	0,5	0,25	0,5	0,5
ATCC-27853	0,5 – 4*	1	1	1	1	1
CSR-55	4 – 16	16	8	8	8	8
CSR-57	1 – 4	4	4	2	4	4
CSR-68	8 – 32	≥16	≥16	≥16	≥16	≥16
NCTC-13846	2 – 8*	4	16	4	4	8
Negative control		NG	NG	NG	NG	NG
	MIC: expected values (range)	panels stored at RT				
		T0	T7	T14	T30	T90
ATCC-25922	0,25 – 2*	0,5	0,5	0,5	1	1
ATCC-27853	0,5 – 4*	1	1	1	1	1
CSR-55	4 – 16	8	16	8	16	8
CSR-57	1 – 4	4	4	2	4	4
CSR-68	8 – 32	≥16	≥16	≥16	≥16	≥16
NCTC-13846	2 – 8*	4	4	4	4	4
Negative control		NG	NG	NG	NG	NG
	MIC: expected values (range)	panels stored at 35°C				
		T0	T7	T14	T30	T90
ATCC-25922	0,25 – 2*	0,5	0,5	0,5	1	1
ATCC-27853	0,5 – 4*	1	1	1	1	2
CSR-55	4 – 16	8	8	8	8	≥16
CSR-57	1 – 4	4	4	4	4	4
CSR-68	8 – 32	≥16	≥16	≥16	≥16	≥16
NCTC-13846	2 – 8*	4	4	4	4	8
Negative control		NG	NG	NG	NG	NG

320

321 Table 2 – Stability tests using panels stored at different temperatures in a 3-month period. MICs
322 values for the different strains are shown. *: according to EUCAST Routine and Extended Internal
323 Quality Control for MIC Determination and Disk Diffusion (version 7.0, valid from 2017-01-01). NG
324 = no growth

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	MIC: expected values (range)	single panel replicates, different days			
		T0	T5	T13	T19
ATCC-25922	0,25 – 2*	0,5	0,5	1	1
ATCC-27853	0,5 – 4*	1	1	2	2
CSR-55	4 – 16	16	8	8	8
CSR-57	1 – 4	4	4	4	4
CSR-68	8 – 32	>=16	>=16	>=16	>=16
NCTC-13846	2 – 8*	4	4	4	4

Table 3 - Stability tests using the same panel inoculated in different days. *: according to EUCAST Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion (version 7.0, valid from 2017-01-01).

Figure 1 – Left part: results of a STC test. The well on the right of the red line indicates the value of the MIC for the isolate. Right part: STC test, with arrows indicating pinpoint colonies of *H. alvei*.

Figure 2 – Evaluation of the bactericidal effect by sampling of 1 ml of the resuspended wells of a strain having an MIC of 8 mg/ml (MIC = MBC).

REFERENCES

1. **Koyama Y, Kurosasa A, Tsuchiya A, Takakuta K.** 1950. A new antibiotic 'colistin' produced by spore-forming soil bacteria. J. Antibiot. **3:**457-458.
2. **Landman D, Georgescu C, Martin DA, Quale J.** 2008. Polymyxins revisited. Clin Microbiol Rev **21:**449-465.

3. **Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL.** 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis **6:**589-601.

4. **Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL.** 2011. Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. Antimicrob Agents Chemother **55:**3284-3294.

5. **Jeannot K, Bolard A, Plesiat P.** 2017. Resistance to polymyxins in Gram-negative organisms. Int J Antimicrob Agents **49:**526-535.

6. **Kato A, Latifi T, Groisman EA.** 2003. Closing the loop: the PmrA/PmrB two-component system negatively controls expression of its posttranscriptional activator PmrD. Proc Natl Acad Sci U S A **100:**4706-4711.

7. **Lee JY, Park YK, Chung ES, Na IY, Ko KS.** 2016. Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. Sci Rep **6:**25543.

8. **Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Hoiby N.** 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother **56:**1019-1030.

9. **Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J.** 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis **16:**161-168.

10. **Schwarz S, Johnson AP.** 2016. Transferable resistance to colistin: a new but old threat. J Antimicrob Chemother **71:**2066-2070.

11. **Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S.** 2016. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. Euro Surveill **21**.

12. **Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Shen J, Wang Y.** 2017. Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in Escherichia coli. MBio **8**.

13. **Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF.** 2017. Novel plasmid-mediated colistin resistance mcr-4 gene in Salmonella and Escherichia coli, Italy 2013, Spain and Belgium, 2015 to 2016. Euro Surveill **22**.

14. **Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B.** 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B. J Antimicrob Chemother.

15. **EUCAST.** 2016. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf.

16. **EUCAST.** 2016. Intrinsic Resistance and Exceptional Phenotypes Tables - EUCAST Expert Rules Version 3.1. http://www.eucast.org/expert_rules_and_intrinsic_resistance/.

17. **Jayol A, Saly M, Nordmann P, Menard A, Poirel L, Dubois V.** 2017. Hafnia, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. J Antimicrob Chemother.

18. **Wang Y, Tian GB, Zhang R, Shen Y, Tyrrell JM, Huang X, Zhou H, Lei L, Li HY, Doi Y, Fang Y, Ren H, Zhong LL, Shen Z, Zeng KJ, Wang S, Liu JH, Wu C, Walsh TR, Shen J.** 2017. Prevalence, risk factors, outcomes, and molecular epidemiology of mcr-1-positive Enterobacteriaceae in patients and healthy adults from China: an epidemiological and clinical study. Lancet Infect Dis **17:**390-399.

19. **Caniaux I, van Belkum A, Zambardi G, Poirel L, Gros MF.** 2017. MCR: modern colistin resistance. Eur J Clin Microbiol Infect Dis **36:**415-420.

20. **EUCAST.** 2017. Antimicrobial susceptibility testing of colistin - Problems detectd with several commercially available products. www.eucast.org/ast_of_bacteria/warnings/#c13111.

21. **Chew KL, La MV, Lin RTP, Teo JWP.** 2017. Colistin and Polymyxin B Susceptibility Testing for Carbapenem-Resistant and mcr-Positive Enterobacteriaceae: Comparison of Sensititre, MicroScan, Vitek 2, and Etest with Broth Microdilution. J Clin Microbiol **55:**2609-2616.

22. **CLSI.** 2016. Performance standards for antimicrobial susceptibility testing. 26th Edition. CLSI document M100-S. Wayne, PA: Clinical and Laboratory Standards Institute.

23. **FDA.** 2009. Class II Special Controls Guidance Document: Antimicrobial Susceptibility Test (AST) Systems.1-42.