AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

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An Improved Medium for Colistin Susceptibility Testing

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ABSTRACT The plasmid-located colistin resistance gene *mcr-1* confers low-level resistance to colistin, a last-line antibiotic against multidrug-resistant Gram-negative bacteria. Current CLSI-EUCAST recommendations require the use of a broth microdilution (BMD) method with cation-adjusted Mueller-Hinton (CA-MH) medium for colistin susceptibility testing, but approximately 15% of all MCR-1 producers are classified as sensitive in that broth. Here we report on an improved calcium-enhanced Mueller-Hinton (CE-MH) medium that permits simple and reliable determination of *mcr-1*-containing *Enterobacteriaceae*. Colistin susceptibility testing was performed for 50 *mcr-1*-containing *Escherichia coli* and *Klebsiella pneumoniae* isolates, 7 intrinsically polymyxin-resistant species, *K. pneumoniae* and *E. coli* isolates with acquired resistance to polymyxins due to *mgrB* and *pmrB* mutations, respectively, and 32 *mcr-1*-negative, colistin-susceptible isolates of *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium. A comparison of the colistin MICs determined in CA-MH medium and those obtained in CE-MH medium was performed using both the BMD and strip-based susceptibility test formats. We validated the data using an isogenic IncX4 plasmid lacking *mcr-1*. Use of the CE-MH broth provides clear separation between resistant and susceptible isolates in both BMD and gradient diffusion assays; this is true for both *mcr-1*-containing *Enterobacteriaceae* isolates and those exhibiting either intrinsic or acquired colistin resistance. CE-MH medium is simple to prepare and overcomes current problems associated with BMD and strip-based colistin susceptibility testing, and use of the medium is easy to implement in routine diagnostic laboratories, even in resource-poor settings.

KEYWORDS colistin susceptibility testing, *mcr-1* resistance, calcium ions

Polymyxins, which include polymyxin B and colistin, are pentacationic lipopeptide antibiotics produced by the bacterium *Paenibacillus polymyxa* (1). In analogy to polymyxin B, colistin selectively binds to the lipopolysaccharide (LPS) of Gram-negative bacteria. It competitively displaces divalent cations (such as Ca^{2+} and Mg^{2+}) that bridge adjacent LPS molecules, and insertion of its lipopeptide moiety induces expansion of the outer membrane and, with time, loss of physical integrity of the phospholipid bilayer of the inner membrane. The increase in the permeability of the cell membranes leads to leakage of intracellular contents and ultimately bacterial death (2–6).

Colistin has been used for decades as an oral drug in veterinary medicine, particularly to treat infections associated with pathogenic *Escherichia coli* and *Salmonella* spp. in pig and veal calf herds (7, 8). With the emergence of multidrug-resistant (MDR) Gram-negative bacteria exhibiting resistance to third-generation cephalosporins and carbapenems, colistin is now also used as a last-line antibiotic for treatment of health care-associated infections (9, 10).

Resistance to colistin generally involves mutations in chromosomal genes. Intrinsic resistance to colistin in strains such as *Morganella morganii*, *Proteus mirabilis*, *Proteus*

Received 12 December 2017 **Returned for modification** 15 January 2018 **Accepted** 1 March 2018

Accepted manuscript posted online 7 March 2018

Citation Gwozdzinski K, Azarderakhsh S, Imirzalioglu C, Falgenhauer L, Chakraborty T. 2018. An improved medium for colistin susceptibility testing. J Clin Microbiol 56:e01950-17. https://doi.org/10.1128/JCM.01950-17.

Editor Nathan A. Ledeboer, Medical College of Wisconsin

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vulgaris, *Providencia rettgeri*, and *Serratia marcescens* strains is due to modification of lipopolysaccharide with amino sugars such as L-Ara4N or overexpression of outer membrane proteins. Acquired resistance to polymyxins in strains such as *Klebsiella pneumoniae* and *E. coli* strains involves mutations in the PmrAB/PhoPQ two-component sensing systems or alterations of the *mgrB* and *pmrB* genes (11–13).

Recently, a plasmid-borne *mcr-1* gene conferring resistance to colistin that could be transmitted by horizontal gene transfer was identified (14). This new mechanism could contribute to increased occurrence of colistin resistance. The degree of resistance conferred by *mcr-1* is lower than that of the other colistin resistance mechanisms encoded on the chromosome (15).

Current antimicrobial susceptibility testing for colistin is fraught with pitfalls, and a joint CLSI-EUCAST Polymyxin Breakpoints Working Group recently provided warnings regarding the credibility of methods used to determine the MICs of colistin-resistant isolates (16, 17). Currently, it is recommended that the broth microdilution (BMD) method should be used as a standard format for colistin antimicrobial susceptibility testing, but it has been claimed that BMD is time-consuming and requires trained technical staff for manual preparation of antibiotic solutions and assay assessment (18). Some of these difficulties are overcome by the rapid polymyxin NP test, but failure to detect polymyxin-resistant *E. coli* and *Enterobacter* sp. strains using this assay has been reported (19).

The difficulties with colistin resistance testing are associated with the physical properties of cationic lipopeptides, including their poor agar diffusion characteristics and the unidentified conditions required for the activity of MCR-1. Limited laboratory screening procedures using cation-adjusted Mueller-Hinton (CA-MH) medium for BMD have implications for public health and lead to underestimation of the true prevalence of *mcr-1* and thus to possible treatment failures due to methodology-based nondetection of resistance. Therefore, there is a need for a simple assay that promotes reliable and reproducible susceptibility testing using different formats, in order to implement preventive measures (20).

Here we report on a novel medium for the improved determination of *mcr-1*-producing *Enterobacteriaceae*. The broth described here allows reliable monitoring of MCR-1-dependent activity and requires optimized levels of Ca^{2+} for the detection of colistin resistance in *mcr-1*-containing bacteria. The medium is easy to prepare and usable in several assay formats (e.g., microdilution and gradient diffusion) and therefore is a useful addition to protocols currently used for testing of colistin resistance.

(Konrad Gwozdzinski performed this work as a partial fulfillment of the requirements for a Ph.D. at the Faculty of Medicine, Justus Liebig University, Giessen, Germany, 2018. Saina Azarderakhsh performed this work as a partial fulfillment of the requirements for an M.Sc. at the Faculty of Biology, Philipps University of Marburg, Marburg, Germany, 2017.)

MATERIALS AND METHODS

Isolates used in the study. We included 50 *mcr-1*-containing isolates of *E. coli* and *K. pneumoniae* (21), 7 isolates of intrinsically colistin-resistant species, including *M. morganii*, *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, and *S. marcescens*, 8 isolates of *Acinetobacter baumannii*, *E. coli*, or *K. pneumoniae* exhibiting acquired resistance to polymyxins, including 1 isolate of *K. pneumoniae* with a mutation in the *mgrB* gene and 1 isolate of *E. coli* with a mutated *pmrB* gene (D149Y), 32 *mcr-1*-negative and colistin-susceptible isolates representing diverse bacterial species, such as *A. baumannii*, *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium, and 5 *E. coli* J53-DH10β transconjugants with *mcr-1* on either IncX4 or IncHI2 plasmids (22). Details of the relevant characteristics of the isolates are provided in Table 1.

Mutagenesis of the *pmrB* gene. A mutation in the *pmrB* gene of *E. coli* MG1655 leading to activation of the *arnBCADTEF-pmrE* operon (23) was introduced through a plasmid-based portable multiplex automated genome engineering (pORTMAGE) approach, as described previously (24). The sequence of the oligonucleotide carrying a G445T substitution is shown in Table SA4 in the supplemental material. The pORTMAGE plasmid was maintained by addition of 100 mg/liter ampicillin (Sigma-Aldrich) to the medium. For verification of the mutated gene, whole-genome DNA was isolated using the PureLink genome DNA minikit (Thermo Fischer Scientific, Langenselbold, Germany), following the manufacturer's instructions. A Nextera XT library of the genome (Illumina, San Diego, CA, USA) was sequenced with a

TABLE 1 Properties of isolates included in the study

Strain	Source	Incompatibility group of *mcr-1*-positive plasmids[a]	MIC (mg/liter)[b]			
			BMD		Agar diffusion	
			CA-MH	CE-MH	CA-MH	CE-MH
A. baumannii 01[c]	Human	NA	1	2	1.5	1.5
A. baumannii 02[c]	Environment	NA	2	2	1	2
A. baumannii 03[c]	Environment	NA	1	2	2	2
A. baumannii 04[c]	Environment	NA	2	2	1.5	2
A. baumannii 05[c]	Environment	NA	1	1	1	1.5
A. baumannii 06[c]	Environment	NA	1	2	1.5	2
A. baumannii 07[c]	Environment	NA	1	4	1.5	2
A. baumannii 08[c]	Environment	NA	1	4	1	2
A. baumannii 09[c]	Environment	NA	0.5	1	1	2
A. baumannii 010[c]	Environment	NA	1	4	0.75	1.5
C. freundii 01[c]	Human	NA	0.5	1	1	1
C. freundii 02[c]	Human	NA	0.5	1	1	1
E. cloacae 01[c]	Human	NA	1	1	1	1
E. coli ATCC 25922[c]	CLSI QC strain	NA	1	1	1	1.5
E. coli 010[c]	Poultry	NA	1	1	2	2
E. coli 017[c]	Companion animal	NA	0.5	0.5	1.5	1.5
E. coli CAE02[c]	Cattle	NA	1	1	1.5	1.5
E. coli CAE07[c]	Cattle	NA	1	1	1.5	1.5
E. coli CAE13[c]	Cattle	NA	1	1	2	2
E. coli CLO28[c]	Poultry	NA	1	1	1.5	1.5
E. coli CLO29[c]	Poultry	NA	1	2	2	1.5
E. coli CLO47[c]	Cattle	NA	2	2	2	2
E. coli DO14[c]	Companion animal	NA	0.5	0.5	1.5	1.5
E. coli DO21[c]	Companion animal	NA	0.5	0.5	1.5	1.5
E. coli DH10β[c]	Laboratory strain	NA	2	2	2	2
E. coli MG1655[c]	Laboratory strain	NA	2	2	2	2
E. coli J53[c]	Laboratory strain	NA	2	2	2	2
K. pneumoniae S01[c]	Human	NA	1	2	1	1
K. pneumoniae S02[c]	Human	NA	1	1	1	1
K. pneumoniae S03[c]	Human	NA	1	1	1	1
S. Typhimurium ATCC 14028[c]	Human	NA	1	1	1	1
E. coli NCTC 13846[d]	CLSI QC strain	ND	4	32	4	6
E. coli 001[d]	Human	IncHI2	4	32	2	24
E. coli 002[d]	Pig	IncX4	2	32	2	12
E. coli 003[d]	Pig	IncHI2	16	64	4	16
E. coli 004[d]	Pig	IncHI2	16	64	4	24
E. coli 005[d]	Food (beef)	IncX4	8	32	2	16
E. coli 006[d]	Food (poultry)	IncX4	8	32	2	16/24
E. coli 013[d]	Cattle	IncHI2	32	64	4	32
E. coli 018[d]	Food (poultry)	Chromosomal	6	32	2	16
E. coli 020[d]	Food (poultry)	IncHI2	4	16	3	12
E. coli 021[d]	Pig	IncX4	8	64	6	32
E. coli 022[d]	Pig	IncX4	6	32	3	16
E. coli 023[d]	Pig	IncX4	6	32	3	24
E. coli 024[d]	Pig	ND	8	32	3	24
E. coli 025[d]	Pig	IncHI2	6	64	4	24
E. coli 026[d]	Pig	IncHI2	6	32	3	24
E. coli 027[d]	Pig	IncX4	6	32	3	24
E. coli 028[d]	Pig	IncHI2	6	32	3	12
E. coli 029[d]	Pig	IncX4	8	16	3	24
E. coli 030[d]	Pig	IncX4	8	32	4	16
E. coli 031[d]	Food (poultry)	IncHI2	8	32	4	16
E. coli 032[d]	Food (poultry)	IncHI2	16	32	4	16
E. coli 033[d]	Food (poultry)	IncX4	6	32	3	16
E. coli 034[d]	Food (poultry)	ND	8	32	4	24
E. coli 035[d]	Food (poultry)	ND	8	64	4	24
E. coli 037[d]	Pig	IncX4	4	8	3	6
E. coli 038[d]	Pig	IncX4	6	64	4	24
E. coli 039[d]	Pig	IncHI2	6	32	3	16
E. coli 040[d]	Pig	IncX4	6	32	4	12
E. coli 041[d]	Pig	IncX4	4	16	4	16

(Continued on next page)

TABLE 1 (Continued)

Strain	Source	Incompatibility group of *mcr-1*-positive plasmids[a]	MIC (mg/liter)[b]			
			BMD		Agar diffusion	
			CA-MH	CE-MH	CA-MH	CE-MH
E. coli 042[d]	Pig	IncX4	6	32	4	24
E. coli 043[d]	Pig	IncHI2	6	32	4	12
E. coli 044[d]	Pig	IncHI2	8	32	4	24
E. coli 045[d]	Pig	ND	8	64	6	24
E. coli 046[d]	Pig	IncX4	6	32	3	16
E. coli 047[d]	Pig	IncX4	6	32	4	16
E. coli 048[d]	Pig	IncX4	6	32	3	24
E. coli 049[d]	Pig	IncX4	6	32	4	16
E. coli 050[d]	Pig	IncX4/IncN	8	32	4	16
E. coli 051[d]	Pig	IncX4	6	32	4	16
E. coli 052[d]	Pig	IncX4	8	64	6	24
E. coli 053[d]	Pig	ND	8	32	4	24
E. coli 054[d]	Poultry	IncHI2	8	32	4	16
E. coli 055[d]	Poultry	ND	4	32	4	24
E. coli 056[d]	Poultry	IncX4	16	64	6	24
E. coli 057[d]	Poultry	IncX4	8	64	4	24
E. coli 058[d]	Poultry	ND	32	128	6	32/48
E. coli 059[d]	Poultry	IncX4	4	32	4	24
E. coli 060[d]	Poultry	IncHI2	8	32	4	16
K. pneumoniae 002[d]	Poultry	ND	>128	>128	>128	>128
p002/DH10β[d]	Transconjugant	IncX4	4	32	4	32
p002::Δ*mcr-1*[c]	Δ*mcr-1* of p002 in *E. coli* DH10β	IncX4	2	2	2	2
t004/J53[d]	Transconjugant of *E. coli* J53 harboring p004	IncHI2	6	32	2	16
t002/J53[d]	Transconjugant of *E. coli* J53 harboring p002	IncX4	8	64	2	24
t003/J53[d]	Transconjugant of *E. coli* J53 harboring p003	IncHI2	8	64	2	12
t001/J53[d]	Transconjugant of *E. coli* J53 harboring p001	IncHI2	6	64	2	12
Klebsiella pneumoniae[e]	Human	NA	4	8	4	6
E. coli MG1655 *pmrB* D149Y[f]	Laboratory strain	NA	8	32	3	8
K. pneumoniae R01	Human	NA	64	32	8	32
K. pneumoniae R02	Human	NA	64	32	3	6
K. pneumoniae R03	Human	NA	64	>128	32	>128
Morganella morganii 5174[g]	Human	NA	>128	>128	16	>128
Proteus mirabilis 519[g]	Human	NA	>128	>128	>128	>128
Proteus mirabilis 5242[g]	Human	NA	>128	>128	>128	>128
Proteus vulgaris 5243[g]	Human	NA	>128	>128	>128	>128
Providencia rettgeri 5198[g]	Human	NA	>128	>128	>128	>128
Serratia marcescens 16029566[g]	Human	NA	>128	>128	>128	>128
Serratia marcescens 340547[g]	Human	NA	16	>128	8	>128
Serratia marcescens 5149[g]	Human	NA	>128	>128	8	>128

[a]NA, not applicable; ND, not determined.
[b]Colistin MIC values were determined by both broth microdilution and strip-based methods, in CA-MH broth and CE-MH broth. False-susceptible isolates that were resistant with the test method (BMD in CE-MH broth) and susceptible with the reference method (BMD in CA-MH broth) are highlighted in gray.
[c]*mcr-1*-negative, colistin-susceptible isolates.
[d]*mcr-1*-positive isolates.
[e]Mutation in the *mgrB* gene involved in colistin resistance.
[f]Mutation in the *pmrB* gene (D149Y) involved in colistin resistance.
[g]Intrinsically polymyxin-resistant species.

MiSeq system (using 2 by 300 cycles). Raw data were assembled using SPAdes (25), and comparison with the wild-type *pmrB* gene was performed using BLASTn.

Preparation of inactivated human serum samples. For normal human serum (NHS) collection, a consenting adult volunteer donated blood. After clotting at room temperature, the NHS component was aseptically harvested and used fresh. NHS was heated for 30 min at 56°C in order to inactivate complement.

Preparation of calcium-enhanced Mueller-Hinton broth. The medium devised here is based on Mueller-Hinton (MH) broth supplemented with calcium chloride dehydrate, i.e., calcium-enhanced Mueller-Hinton (CE-MH) broth. To prepare 1 liter of CE-MH medium, 23 g of MH broth powder (product no. 70192; Sigma-Aldrich, Darmstadt, Germany) was dissolved in 1 liter of distilled water and sterilized by autoclaving at 121°C for 15 min. After the solution was cooled to room temperature, 5 ml of a sterile-filtered 1 M stock solution of calcium chloride dihydrate (product no. 5239.3; Carl Roth, Karlsruhe, Germany) was added to the MH broth to achieve a final molarity of 5 mM in the medium. For solid media, 15 g/liter agar was added before autoclaving. Storage of MH agar plates supplemented with 5 mM calcium chloride dihydrate for up to 4 weeks at 4°C did not affect strip-based colistin susceptibility

testing. The reference medium, CA-MH broth, was prepared from MH broth supplemented with Ca^{2+} and Mg^{2+}, according to CLSI-EUCAST guidelines (16).

MIC determination. BMD to determine the MICs of the isolates was performed according to the recommendations of the joint CLSI-EUCAST Polymyxin Breakpoints Working Group (16). A range of 0.25 to 128 mg/liter colistin sulfate (Sigma-Aldrich) was tested. Plain 96-well polystyrene microplates (Greiner, Frickenhausen, Germany) were used for broth microdilution experiments. Each isolate was examined for growth in either CA-MH or CE-MH medium. MIC determination was performed by visual inspection following overnight incubation of the plate at 37°C.

MIC determination by the strip-based method was performed in accordance with the manufacturer's instructions (product no. 01B10093; Liofilchem, Roseto degli Abruzzi, Italy). The MIC was read at 1 dilution step above the area where the growth inhibition area intersected with the antibiotic strip. When small colonies grew within the area of inhibition or a haze of growth occurred around the MIC endpoint, the highest MIC intersection was noted. For MIC determination including serum, CA-MH medium was supplemented with 20% heat-inactivated NHS.

Interpretation of MIC data. We used the EUCAST breakpoint values for polymyxins as a reference (26). Enterobacterial isolates with colistin MICs of $\leq$2 mg/liter were categorized as susceptible, and those with MICs of $>$2 mg/liter were categorized as resistant.

Precision and reproducibility testing of CE-MH broth. Three isolates (*E. coli* 010, *E. coli* CLO28, and *mcr-1*-expressing *E. coli* 051) were chosen at random and tested according to the requirements described in the CLSI document for development of *in vitro* susceptibility testing criteria and quality control (QC) parameters (27). Three replicates using individual inoculum preparations of the appropriate strains were tested for 5 consecutive test days, using the 15-replicate (3 by 5 days) plan. These data were used to assess the interassay and intra-assay precision and reproducibility of the MICs obtained in CE-MH medium with the BMD and agar-based methods. On each testing day, the CLSI-recommended *E. coli* ATCC 25922 and *E. coli* NCTC 13846 QC strains were included. All of the MICs determined were within the acceptable QC range.

Deletion of the *mcr-1* gene from p002. The *mcr-1* deletion mutant of p002 (*mcr-1*-containing IncX4 plasmid of *E. coli* 002) (22) (Fig. SA2), p002::Δ*mcr-1*, was generated using the primers listed in Table SA5, as described previously (28). Plasmids were maintained by the addition of 2 mg/liter colistin sulfate salt (Sigma-Aldrich) or 30 mg/liter kanamycin (Sigma-Aldrich) to the broth. The complete sequence of p002 was determined using long-read single-molecule real-time (SMRT) sequencing (Pacific Biosciences, Menlo Park, CA, USA) supplemented with short-read sequencing using the Illumina platform, as described previously (29). For sequencing of p002::Δ*mcr-1*, plasmid DNA was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. A Nextera XT library of the plasmid (Illumina) was sequenced on the NextSeq 500 platform (using 2 by 150 cycles). Raw data were assembled using SPAdes (25), and comparison of p002 with p002::Δ*mcr-1* was performed using BLASTn.

Statistical analysis. In our study, CA-MH broth was the reference medium. Categorical agreement and essential agreement (EA) between CA-MH and CE-MH media for both BMD and strip-based methods were calculated according to ISO guidelines (30) and by using kappa scores (31). Categorical agreement is defined as the MIC from the test that is in the same susceptibility category as the currently used reference method. EA is defined as the MIC obtained for the test that is within $\pm$1 twofold dilution of the currently used reference method. Statistical analysis was performed using the MedCalc software package (MedCalc, Mariakerke, Belgium) and Excel (Microsoft, Redmond, CA, USA).

Accession number(s). The plasmid sequences of p002 and p002::Δ*mcr-1* were deposited in the GenBank database under accession numbers MF381176 and MF381175, respectively.

RESULTS

A preliminary analysis of the colistin MIC values revealed that *mcr-1*-containing *Enterobacteriaceae* isolates exhibited increased colistin resistance when grown in the presence of heat-inactivated NHS (see Table SA1 in the supplemental material). Because serum contains high levels of calcium ions (32), we assessed whether Ca^{2+} added to growth media could be used as an enhancing agent for the detection of *mcr-1*-containing *Enterobacteriaceae*. We determined that elevated concentrations of Ca^{2+} in the growth medium are optimal for the unambiguous detection of *mcr-1*-positive Gram-negative bacteria.

We devised a novel formulation, called calcium-enhanced Mueller-Hinton (CE-MH) broth, which contains 200 mg/liter Ca^{2+} ions (5 mM), and we compared it to the reference medium (CA-MH), employing microdilution and strip-based methods. The concentration of calcium used in CE-MH broth had no adverse effects on bacterial growth (Fig. SA1).

We examined 50 *mcr-1*-positive isolates by broth microdilution and observed a pronounced difference in MIC values between CA-MH broth and the CE-MH broth devised here. The colistin MICs ranged from 4 mg/liter to 8 mg/liter in CA-MH broth and increased to 16 to 32 mg/liter in CE-MH broth (Table 1). Polymyxin-sensitive isolates lacking *mcr-1* remained susceptible to colistin when examined in CE-MH medium. The

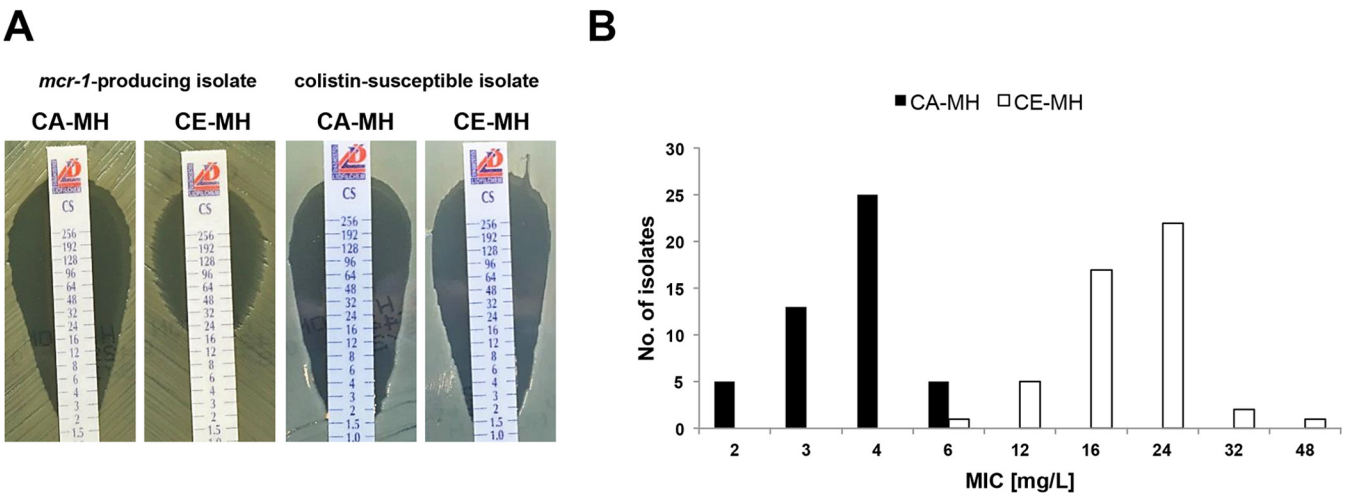

FIG 1 Effects of an elevated calcium concentration on the colistin MICs of *mcr-1*-containing isolates, as determined in cation-adjusted Mueller-Hinton (CA-MH) broth and calcium-enhanced Mueller-Hinton (CE-MH) broth by the agar diffusion method. (A) Colistin MICs of representative colistin-susceptible and *mcr-1*-positive isolates. (B) Susceptibility testing of 50 isolates containing the *mcr-1* gene. A significant upward shift in colistin MIC values was observed in CE-MH medium, compared to the reference CA-MH medium.

exceptions were 3 *A. baumannii* isolates that were classified as susceptible by the reference method and as resistant by BMD in CE-MH broth (Table 1); this was not observed when the agar diffusion format was used.

Whole-genome sequencing performed for the *A. baumannii* isolates revealed mutations in the *pmrC* gene in the isolates (*pmrC* I228V and R348K for *A. baumannii* 07, V231A and A401V for *A. baumannii* 08, and H36Q, A55V, and A401G for *A. baumannii* 010), compared with the colistin-susceptible strain *A. baumannii* ATCC 17978. This suggests improved detection of colistin-resistant *A. baumannii* with BMD performed in CE-MH medium.

We next examined the usefulness of the medium in an agar-based diffusion format (strip test). A significant increase in colistin MIC values was also noted with CE-MH medium, compared to the reference CA-MH medium, when strips were used on CA-MH agar plates (Fig. 1 and Table 1). Isolates that were intrinsically resistant to colistin, such as *M. morganii*, *Proteus* sp., *P. rettgeri*, and *S. marcescens* isolates, and isolates that had acquired resistance due to mutations in the *mgrB* gene (*K. pneumoniae*) or the *pmrB* gene (*E. coli*) also exhibited increased MIC values with CE-MH medium (Table 1). Thus, CE-MH medium clearly detects all classes of colistin-resistant bacteria, particularly *mcr-1*-containing isolates, regardless of the assay format (liquid or solid) used. However, isolates of *A. baumannii* remain a challenge when CE-MH medium is used in an agar diffusion format.

To directly compare the resistance levels of isolates harboring *mcr-1* on IncX4 or IncHI2 plasmids, we used transconjugants of *E. coli* J53 harboring either plasmid. As before, we observed increased colistin MIC values for transconjugants grown in CE-MH medium, compared to CA-MH medium, as determined by both agar diffusion and microdilution methods. The colistin MIC values were independent of the incompatibility group of the plasmid used (Table 1).

To confirm the contribution of the *mcr-1* gene to calcium-induced resistance to colistin, the *mcr-1* gene was deleted from the IncX4 plasmid p002 to create the isogenic plasmid variant p002::Δ*mcr-1*. The colistin MICs of *E. coli* DH10β containing the wild-type p002 or p002::Δ*mcr-1* were determined in CA-MH medium to be 4 mg/liter and 2 mg/liter, respectively. When tested in CE-MH medium, the MIC of the strain with p002 increased to 32 mg/liter, while that of the strain with p002::Δ*mcr-1* remained 2 mg/liter, indicating an essential role of the *mcr-1* gene in calcium-induced colistin resistance (Table 2).

The comparison of the MIC values obtained in CA-MH and CE-MH media demon-

TABLE 2 Colistin susceptibility testing results for *E. coli* DH10β harboring p002 or p002::Δ*mcr-1*, as determined by broth microdilution in CA-MH broth and CE-MH broth

Strain	MIC (mg/liter)[a]	
	CA-MH	CE-MH
E. coli DH10β	2	2
p002 in DH10β	4	32
p002::Δ*mcr-1* in DH10β	2	2

[a]The MIC is defined as the lowest concentration of colistin that inhibits visible growth of the tested isolate, as observed with the unaided eye.

strated almost identical essential and categorical agreements for colistin-susceptible isolates, ranging from slight to almost perfect agreement for colistin-resistant isolates using both BMD and agar-based methods (Table SA2). For colistin-susceptible isolates, the essential agreement (EA) values calculated for the BMD and strip-based methods were 91% (29/32 isolates [95% confidence interval [CI], 83 to 96%]) and 100% (32/32 isolates [95% CI, 96 to 100%]), respectively. For colistin-resistant isolates, the EA values for the BMD and strip-based methods were 24% (16/68 isolates [95% CI, 12 to 41%]) and 13% (9/68 isolates [95% CI, 5 to 34%]), respectively. Essential agreement values obtained for colistin-resistant isolates were significantly lower than the EA values for colistin-sensitive isolates as colistin MICs in CE-MH medium. The categorical agreement values (test results with correct susceptibility categorization) for colistin-resistant and colistin-susceptible isolates varied from 91% to 99% for the BMD method and from 87% to 100% for the gradient test method.

Precision and reproducibility results were within the acceptable ranges for 2 colistin-susceptible isolates and 1 colistin-resistant isolate of *E. coli*, as observed with BMD and agar-diffusion-based methods in CE-MH medium (Table SA3). For the QC strains, i.e., colistin-susceptible *E. coli* ATCC 25922 and colistin-resistant, *mcr-1*-positive *E. coli* NCTC 13846, the MICs determined with both BMD and gradient tests were within acceptable ranges, ranging from 0.5 to 1.5 mg/liter and from 6 to 32 mg/liter, respectively.

DISCUSSION

The simple and accurate phenotypic detection of colistin resistance mediated by the horizontally transferable *mcr-1* gene in *Enterobacteriaceae* remains a challenge for routine microbiology laboratories determining antibiotic susceptibilities. The *mcr-1* gene is highly conserved and is capable of conferring resistance to colistin but clearly only under defined conditions. Thus, even though the presence of the *mcr-1* gene results in increased resistance to polymyxin antibiotics, many isolates exhibit colistin MICs of 2 mg/liter, just below the EUCAST clinical breakpoint, and therefore are categorized as sensitive despite harboring the plasmid-borne colistin resistance gene (15). This prompted a joint CLSI-EUCAST subcommittee to issue warnings related to the overall poor quality of colistin susceptibility testing (16). Thus, approaches to simplify and to improve MIC determinations for cationic antimicrobial peptides, e.g., colistin, are highly desirable.

Our studies were prompted by the observation that the presence of heat-inactivated serum increased the colistin MIC values of isolates harboring *mcr-1*. Because the concentration of Ca^{2+} ions in serum is approximately 2.5 mM (32), we used increasing concentrations of these ions to titrate and to devise the medium presented in this study (CE-MH medium). All of the *mcr-1*-containing isolates grown in CE-MH medium exhibited increased MIC values, compared to growth in the reference CA-MH medium. There was a clear separation of MIC values between resistant and susceptible bacteria when CE-MH medium was used.

The CE-MH broth used here has a very important property, i.e., it increases colistin MICs only in isolates that are resistant to polymyxins. Colistin-susceptible isolates that lack *mcr-1* do not display increased MICs when grown in this medium, and the concentration of calcium used has no adverse effects on bacterial growth. The use of a strain harboring an IncX4 plasmid lacking the *mcr-1* gene confirms the contribution

of the gene to calcium-dependent resistance to colistin. The increase in the MIC values for isolates exhibiting intrinsic or acquired resistance to colistin suggests that the elevated level of Ca^{2+} has a general effect, probably due to positively charged modified lipopolysaccharides. The mechanisms underlying the calcium effect remain to be understood but, inasmuch as there is no increased resistance of colistin-sensitive isolates, Ca^{2+} does not antagonize the bactericidal effect of colistin by preventing binding of the peptide to the bacterial cell wall.

False-susceptible and resistant isolates constitute a serious problem for current colistin susceptible testing and consequently for adequate therapy (33). This study included a set of *A. baumannii* isolates that were classified as resistant with our test method (BMD in CE-MH medium) and as susceptible with the reference method (BMD in CA-MH medium) (Table 1). Genome analysis of these isolates demonstrated specific alterations in the sequence of the *pmrCAB* operon, compared to the colistin-sensitive *A. baumannii* ATCC 17978 strain, providing evidence for the efficacy of the CE-MH medium, compared to the reference broth. Further studies with a large and diverse panel of *A. baumannii* isolates will be required to validate these findings.

The essential and categorical agreements between MIC values obtained in CA-MH and CE-MH media with BMD and strip-based methods were highly congruent and ranged from 91% to 100% for colistin-susceptible isolates, indicating that, as an alternative to CA-MH broth, CE-MH medium could also be used for colistin susceptibility testing for *Enterobacteriaceae*. The novelty of this study is that CE-MH medium allows resistance determinations using both solid and liquid formats, without a loss of specificity. This could simplify MIC determinations, for instance by the use of strip tests, a methodology that is widely used in many laboratories and could be again implemented as a suitable method for colistin resistance testing, due to its improved performance with CE-MH agar plates versus CA-MH agar plates (Table 1).

One limitation of this medium is that it does not differentiate between MCR-1 producers and colistin-resistant isolates displaying other colistin resistance mechanisms, i.e., isolates with intrinsic or adaptive colistin resistance, and additional testing for the presence of *mcr-1* by other methods is required. However, this limitation could be easily overcome by supplementing our CE-MH medium with a zinc-chelating agent, such as EDTA (34). A further limitation is that, unlike the rapid polymyxin NP test, which can determine colistin susceptibility in 2 h, the test formats described here require overnight incubation. The real advantage of our formulation is that it enables assays to be performed in extremely simple agar-diffusion-based formats, such as by employing Etests.

The Ca^{2+}-enhanced medium described here will make surveillance studies easier and lead to the development of simpler assays for tracking of *mcr-1*-containing *Enterobacteriaceae*. Routine colistin resistance testing will improve our understanding of the true prevalence of *mcr-1* and help us to devise guidelines and studies to limit its spread. Ultimately, standardized studies, ideally performed under the supervision of internationally authorized committees on antibiotic susceptibility testing, will be required to validate our assay formats, particularly with respect to the levels of skills required to implement the assay and its value in clinical settings and for patient management.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01950-17.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

We thank Christina Gerstmann and Natalia Lest (Institute of Medical Microbiology, Giessen, Germany) for excellent technical assistance.

This study was supported by grants to the German Center of Infection Research (DZIF), through the German Federal Ministry of Education and Research (BMBF) (grant

8000 701-3 [HZI] to T.C. and C.I. and grants TI06.001 and 8032808811 to T.C.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

A European patent form, corresponding to this work, has been filed on behalf of the Justus Liebig University (Giessen, Germany).

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