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A Novel Phenotypic Method To Screen for Plasmid-Mediated Colistin Resistance among *Enterobacteriales*

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ABSTRACT Plasmid-mediated colistin resistance (PMCR), a consequence of the *mcr* genes, is a significant public health concern given its potential to easily spread among clinical pathogens. Recently, it was discovered that MCR enzymes require zinc for activity. Thus, we modified the colistin broth-disk elution (CBDE) test to screen for plasmid-mediated colistin resistance (PMCR) genes based on any reduction of colistin MIC in the presence of EDTA. Eighty-five isolates of the order *Enterobacteriales* (12 *mcr* positive) were tested by CBDE ± EDTA. The sensitivity and specificity of the EDTA-CBDE method to detect PMCR compared to the molecular genotype results were 100% and 95.8%, respectively. Isolates positive by the EDTA-CBDE test should be further evaluated to confirm the presence of *mcr* genes.

KEYWORDS colistin, *mcr*, phenotypic method, plasmid-mediated colistin resistance

The rise of multidrug-resistant Gram-negative infections, especially carbapenem-resistant *Enterobacteriales*, has led to the resurgence of colistin as a last-resort therapy (1). Colistin is a polycationic antimicrobial that disrupts the outer membrane of Gram-negative bacteria by binding to negatively charged lipid polysaccharides (LPS) of the cell wall, causing pore formation and eventually cell death (2). Accordingly, acquired or intrinsic resistance to colistin occurs through the modification or depletion of LPS by reducing the anionic charge of the cell membrane, followed by a subsequent decrease in colistin binding affinity (2). Until recently, all mechanisms of colistin resistance were thought to be mediated by chromosomal mutations (3). However, in 2015, the first plasmid-mediated colistin resistance (PMCR) gene was described in China, and now *mcr-1* and its variants (*mcr-2* to *mcr-8*) have been described globally (3, 4). MCR are metalloproteins that transfer phosphoethanolamine to the lipid A portion of LPS, reducing the overall net negative charge of the cell wall (5). PMCR is a significant public health concern given its potential to spread easily among clinical pathogens (4).

Recently, the colistin broth-disk elution (CBDE) method was described as a simple and accurate method to perform colistin antimicrobial susceptibility testing (6). Furthermore, it was discovered that MCR enzymes require zinc for activity (5). The objective of this study was to further optimize the functionality of the newly described colistin broth-disk elution test used for antimicrobial susceptibility testing of colistin to serve as a screen for PMCR genes based on the reduction of the colistin MIC in the presence of EDTA, a known chelator of zinc (5).

MATERIALS AND METHODS

Eighty-five isolates of the order *Enterobacteriales* were evaluated, including 12 MCR-producing isolates from the Centers for Disease Control and Prevention (CDC) & Food and Drug Administration (FDA) Antimicrobial Resistance (AR) Isolate Bank (www.cdc.gov/arisolatebank/) and 73 clinical

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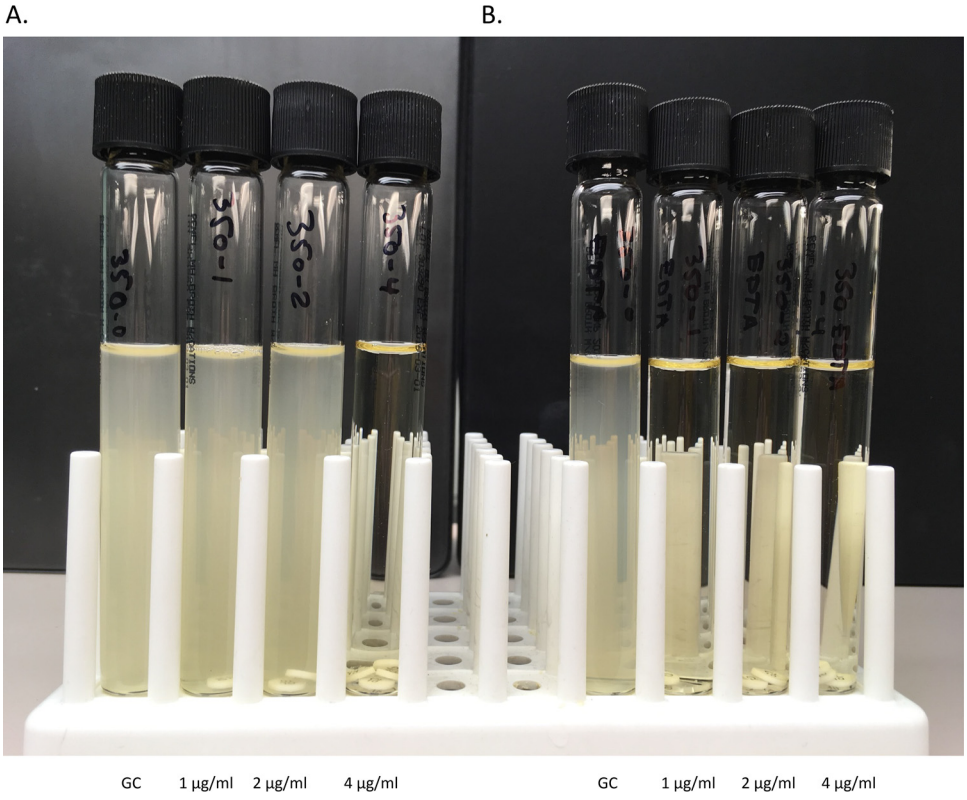

FIG 1 Colistin broth-disk elution and EDTA colistin broth-disk elution methods. Colistin broth-disk elution (CBDE) with (A) and without (B) 1 mM EDTA (EDTA-CBDE). An *mcr-1*-producing *Escherichia coli* isolate (CDC AR Bank number 350) with a colistin MIC of 4 μg/ml based on the CBDE method. The colistin MIC is reduced to $\leq$1 μg/ml in the presence of EDTA, consistent with a positive EDTA-CBDE result and indicating plasmid-mediated colistin resistance. GC indicates growth control.

carbapenem-resistant *Enterobacteriales* lacking *mcr* genes from The Johns Hopkins Hospital Clinical Microbiology Laboratory (see Table S1 in the supplemental material). Illumina MiSeq sequencing (Illumina, San Diego, CA) and/or Nanopore (Oxford, England) whole-genome sequencing results were used to evaluate for the presence of *mcr-1* to *mcr-8* genes among clinical isolates (7).

Colistin MICs were determined by broth microdilution using the Sensititre GNX2F panel (Thermo Fisher) and by the CBDE method, which performs comparably to reference broth dilution methods (see Table S1) (6). CBDE was performed by setting up four 10-ml cation-adjusted Mueller-Hinton broth (CA-MHB) tubes (Remel, Lenexa, KS) per isolate with 0, 1, 2, and 4 colistin disks (10 μg) (BD, Sparks, MD) added to the tubes, generating a final concentration of 0 (growth control), 1, 2, and 4 μg/ml (Fig. 1). The tubes were incubated at room temperature for 30 min, allowing colistin to elute from the disks, after which a 50-μl aliquot of a 0.5 McFarland standard inoculum suspension of the test isolate was added to achieve a final inoculum of 7.5×10^5 CFU/ml (8). To detect PMCR, a second set of tubes was set up as above for the CBDE, to which EDTA (0.5 M EDTA; Sigma) was added as described further below. The final method utilized a concentration of 1 mM EDTA by adding 20 μl of 0.5 M EDTA to each 10-ml CA-MHB tube. Colistin MIC values were read visually, after 18 to 20 h of incubation at 35°C with and without EDTA. Due to the limited doubling dilutions available by the CBDE method, a putative positive for PMCR by the EDTA-CBDE screen was regarded as any reduction in MIC in the presence of EDTA and subsequently compared to the expected molecular result. Any discordant results between the EDTA-CBDE method and the molecular genotype were repeated, and the repeat result was used in the analysis. The EDTA-CBDE results for the *mcr*-positive isolates were repeated in triplicate, and the modal result was used for the analysis. Quality control was performed using *Pseudomonas aeruginosa* ATCC 27853 and an *mcr-1*-producing *Escherichia coli* isolate from the CDC AR Bank number 349 (CBDE MIC of 2 to 4 μg/ml; EDTA-CBDE MIC $\leq$ 1 μg/ml).

The sensitivity and specificity of the EDTA-CBDE for detection of PMCR were determined in comparison to the presence/absence of *mcr* genes based on the molecular characterization of the isolates.

RESULTS

Initially, we tested a subset of nine isolates to verify the concentration of EDTA necessary to lower colistin MICs with the EDTA-CBDE assay without affecting the

TABLE 1 Colistin broth-disk elution with and without EDTA result summary for 85 *Enterobacteriales*

Isolate type	No. of isolates	CBDE colistin MIC (μg/ml) without EDTA (no.)	CBDE with 1 mM EDTA	
			No. (%) of isolates with a reduction of colistin MIC	MIC (μg/ml) doubling dilution reduction (no.)
***mcr*-producing isolates**[c]	12	2 (2)	2 (100)	≥1
		4 (7)	7 (100)	≥2
		>4 (3)	3 (100)	≥2 (2)
				≥3 (1)
CRE[d]	73	≤1 (55)	NA	NA[e]
		2 (2)	3 (16.7)	≥1 (1)[a]
		4 (1)		≥2 (1)[b]
		>4 (15)		≥3 (1)[b]

[a]One *E. cloacae* isolate tested as 2 μg/ml by the CBDE method and as ≤1 μg/ml by the EDTA-CBDE method, resulting in a ≥1 doubling dilution difference.
[b]Two *K. pneumoniae* isolates tested as >4 μg/ml and 4 μg/ml by the CBDE method and as ≤1 μg/ml by the EDTA-CBDE method, resulting in ≥2 and ≥3 doubling dilution differences, respectively.
[c]The 12 MCR-producing isolates included 7 *Escherichia coli* isolates (6 *mcr-1* and 1 *mcr-2*), 2 *Salmonella enterica* serovar Typhimurium isolates (*mcr-3* and *mcr-4*), 1 *Salmonella enterica* serovar Enteritidis isolate (*mcr-1*), and 1 *Salmonella enterica* serovar Oslo isolate (*mcr*-3) (see Table S1 in the supplemental material).
[d]The clinical carbapenem-resistant *Enterobacteriales* (CRE) included 39 *Klebsiella pneumoniae* isolates, 16 *Enterobacter cloacae* complex isolates, 8 *E. coli* isolates, 4 *Citrobacter freundii* isolates, 3 *Serratia marcescens* isolates, 1 *Klebsiella* (formerly *Enterobacter*) *aerogenes* isolate, 1 *Klebsiella oxytoca* isolate, and 1 *Proteus mirabilis* isolate. The CRE were previously characterized for carbapenemase production and included 35 carbapenemase-producing CRE (29 KPC, 3 NDM, 2 KPC and NDM, and 1 NDM and OXA-48) (see Table S1).
[e]NA, not applicable.

growth of non-*mcr*-bearing strains. The CBDE was performed in parallel with the EDTA-CBDE method at concentrations of 1 mM, 2 mM, and 5 mM EDTA. The isolates tested included four isolates with colistin MICs of ≤2 μg/ml, 3 isolates lacking *mcr* with elevated colistin MICs of >4 μg/ml (2 intrinsically resistant *Serratia marcescens* isolates and 1 *Enterobacter cloacae* isolate), and 2 *mcr-1*-producing *E. coli* isolates (CDC AR Bank numbers 346 and 349). For the nine isolates, 1 mM, 2 mM, and 5 mM EDTA resulted in a reduction of colistin MICs for the 2 *mcr-1*-producing isolates. All other results were not impacted by the addition of 1 mM, 2 mM, and 5 mM EDTA. Based on these results, the addition of 1 mM EDTA was further pursued for the EDTA-CBDE method.

To further evaluate the EDTA-CBDE assay using 1 mM EDTA, screens using all 12 *mcr*-bearing isolates and the 73 carbapenem-resistant *Enterobacteriales* were performed (Table 1; see also Table S1 in the supplemental material). All twelve isolates (100%) harboring *mcr* genes showed a reduction in colistin MIC (≥1 to 3 doubling dilutions) when grown with EDTA, while only 3/73 (4.1%) of non-*mcr* strains showed a reduction. Of the carbapenem-resistant *Enterobacteriales* with colistin MICs of >1 μg/ml (*Citrobacter freundii*, *E. cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *S. marcescens*), only *E. cloacae* (1 isolate, 25.0%) and *K. pneumoniae* (2 isolates, 11.1%) showed a reduction in colistin susceptibility in the presence of EDTA. The two *K. pneumoniae* isolates tested as >4 μg/ml and 4 μg/ml by the CBDE method and ≤1 μg/ml by the EDTA-CBDE method, resulting in ≥2 and ≥3 doubling dilution differences, respectively. The *E. cloacae* isolate tested as 2 μg/ml by the CBDE method and ≤1 μg/ml by the EDTA-CBDE method, resulting in a ≥1 doubling dilution difference. The sensitivity and specificity of the CBDE with EDTA technique in detecting PMCR were found to be 100% and 95.8%, respectively.

DISCUSSION

Our study suggests that EDTA-CBDE is an effective approach to screen for the presence of *mcr* utilizing reagents with low cost and high availability. There are a few published methods for detecting PMCR utilizing chelators, such as EDTA or dipicolinic acid (DPA). The EDTA-based combined disk test demonstrated initial promise in *mcr*-bearing *E. coli* isolates, but there are conflicting results about its reliability among *Enterobacteriales* (9, 10). Similarly, a DPA-based disk diffusion test was attempted but performed poorly. These disk-based tests are not reliable due to the low and variable diffusion of colistin from the disks (11). A colorimetric modified rapid polymyxin Nordmann/Poirel test (MPNP) demonstrates satisfactory discrimination within glucose-

fermenting bacteria. However, it requires preparation of specialized reagents, and results interpretation can be subjective due to small color changes (10). Broth microdilution following Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations is the gold standard for determining colistin susceptibility, and the addition of EDTA or DPA to this protocol has been used to screen for PMCR. However, this procedure is more laborious and may be inaccessible to most laboratories (10–13).

The use of EDTA to suppress *mcr* has been validated by several groups, but it is not a specific inhibitor as was observed with 3 carbapenem-resistant isolates of the order *Enterobacteriales* that demonstrated an increase in colistin susceptibility in the presence of EDTA (5, 9, 10). While these strains were negative for *mcr*, we limited our analysis of colistin resistance mechanisms to *mcr* genes. Other mechanisms of colistin resistance are also known to activate phosphoethanolamine transferases (e.g., PmrC), which use similar mechanisms as MCR to transfer phosphoethanolamine to the lipid A portion of the LPS (2, 5). The existence of multiple mechanisms of colistin resistance, especially those that may utilize metalloenzyme activity, may lead to false-positive EDTA-CBDE results. Therefore, this screen must be followed by molecular testing or another validated method to confirm the presence of *mcr*. By definition, all screening tests provide presumptive results where confirmation of positive results is required (8). Additionally, a larger multicenter study utilizing the same isolates and a larger number of resistant and *mcr*-bearing isolates tested across various sites is required to confirm the accuracy and reproducibility of the EDTA-CBDE method.

Here, we have presented a user-friendly method using readily available laboratory supplies for screening PMCR. The addition of EDTA into the CBDE protocol shows selective inhibition of growth in strains harboring *mcr* and provides a basis for further molecular analysis. This test may be implemented in any clinical microbiology laboratory, including those in limited resource settings, for infection control purposes as a practical, rapid screen to counter the expansion of PMCR.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00040-19.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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REFERENCES

1. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. 2012. Colistin: an update on the antibiotic of the 21st century. Expert Rev Anti Infect Ther 10:917–934. https://doi.org/10.1586/eri.12.78.
2. Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643. https://doi.org/10.3389/fmicb.2014.00643.
3. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, 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. https://doi.org/10.1016/S1473-3099(15)00424-7.
4. Sun J, Zhang H, Liu YH, Feng Y. 2018. Towards understanding MCR-like colistin resistance. Trends Microbiol 26:794–808. https://doi.org/10.1016/j.tim.2018.02.006.
5. Hinchliffe P, Yang QE, Portal E, Young T, Li H, Tooke CL, Carvalho MJ, Paterson NG, Brem J, Niumsup PR, Tansawai U, Lei L, Li M, Shen Z, Wang Y, Schofield CJ, Mulholland AJ, Shen J, Fey N, Walsh TR, Spencer J. 2017. Insights into the mechanistic basis of plasmid-mediated colistin resistance from crystal structures of the catalytic domain of MCR-1. Sci Rep 7:39392. https://doi.org/10.1038/srep39392.
6. Simner PJ, Bergman Y, Trejo M, Roberts AA, Marayan R, Tekle T, Campeau S, Kazmi A, Bell D, Lewis S, Tamma PD, Humphries R, Hindler JA. 2018. Two-site evaluation of the colistin broth disk elution test to determine colistin in vitro activity against Gram-negative bacilli. J Clin Microbiol 57:e01163-18. https://doi.org/10.1128/JCM.01163-18.
7. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644. https://doi.org/10.1093/jac/dks261.
8. Clinical and Laboratory Standards Institute. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—11th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
9. Clement M, Budel T, Bernasconi OJ, Principe L, Perreten V, Luzzaro F, Endimiani A. 2018. The EDTA-based disk-combination tests are unreliable for the detection of MCR-mediated colistin-resistance in Enter-

obacteriaceae. J Microbiol Methods 153:31–34. https://doi.org/10.1016/j.mimet.2018.08.008.
10. Esposito F, Fernandes MR, Lopes R, Munoz M, Sabino CP, Cunha MP, Silva KC, Cayo R, Martins W, Moreno AM, Knobl T, Gales AC, Lincopan N. 2017. Detection of colistin-resistant MCR-1-positive Escherichia coli by use of assays based on inhibition by EDTA and zeta potential. J Clin Microbiol 55:3454–3465. https://doi.org/10.1128/JCM.00835-17.
11. Coppi M, Cannatelli A, Antonelli A, Baccani I, Di Pilato V, Sennati S, Giani T, Rossolini GM. 2018. A simple phenotypic method for screening of MCR-1-mediated colistin resistance. Clin Microbiol Infect 24:201.e1–201.e3. https://doi.org/10.1016/j.cmi.2017.08.011.
12. EUCAST. 2017. Breakpoints tables for interpretation MICs and zone diameter, version 7.1.
13. Clinical and Laboratory Standards Institute. 2018. Performance standards for antimicrobial susceptibility testing; 28th Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.