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Evaluation of the NG-Test MCR-1 Lateral Flow Assay and EDTA-Colistin Broth Disk Elution Methods To Detect Plasmid-Mediated Colistin Resistance among Gram-Negative Bacterial Isolates

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ABSTRACT Plasmid-mediated colistin resistance (PMCR) is a global public health concern, given its ease of transmissibility. The purpose of this study was to evaluate two methods for the detection of PMCR from bacterial colonies: (i) the NG-Test MCR-1 lateral flow immunoassay (LFA; NG Biotech, Guipry, France) and (ii) the EDTA-colistin broth disk elution (EDTA-CBDE) screening test method. These methods were evaluated using a cohort of contemporary, clinical Gram-negative bacillus isolates from 3 U.S. academic medical centers (126 isolates of the *Enterobacterales*, 50 *Pseudomonas aeruginosa* isolates, and 50 *Acinetobacter* species isolates; 1 isolate was *mcr* positive) and 12 *mcr*-positive CDC-FDA Antibiotic Resistance (AR) Isolate Bank isolates for which reference broth microdilution colistin susceptibility results were available. Eleven (4.6%) isolates were strongly positive by the MCR-1 LFA, with an additional 8 (3.4%) isolates yielding faintly positive results. The positive percent agreement (PPA) and negative percent agreement (NPA) for MCR-1 detection were 100% and 96.1%, respectively. Upon repeat testing, only a single false-positive MCR-2 producer remained, as the isolates with initially faintly positive results were negative. The EDTA-CBDE screening method had an overall PPA and NPA of 100% and 94.3%, respectively. The NPA for the EDTA-CBDE method was slightly lower at 94.2% with *Enterobacterales*, whereas it was 96.0% with *P. aeruginosa*. The MCR-1 LFA and EDTA-CBDE methods are both accurate and user-friendly methods for the detection of PMCR. Despite the rarity of PMCR among clinical isolates in the United States, these methods are valuable tools that may be implemented in public health and clinical microbiology laboratories to further discern the mechanism of resistance among colistin-resistant Gram-negative isolates and to detect PMCR for infection prevention and control purposes.

KEYWORDS colistin, MCR, methods, plasmid-mediated

Colistin, a cationic polymyxin-class drug, has recently seen a revival of its clinical utility as one of the last-resort antimicrobials for the treatment of multidrug-resistant (MDR) Gram-negative bacterial infections (1–3). Resistance to colistin can be conferred in a myriad of ways, with both chromosome- and plasmid-encoded genes

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having been elucidated. Recently, a family of transmissible, plasmid-borne resistance genes, *mcr* (4, 5), has been described primarily in *Enterobacterales*. The *mcr* genes encode metalloenzymes that transfer phosphoethanolamine to lipid A moieties present in the lipopolysaccharide (LPS) of the bacterial outer membrane, thereby impairing colistin binding and imparting a colistin-resistant phenotype (2, 6–8).

Currently, nine *mcr* genes have been described (*mcr-1* to *mcr-9*) (5, 9), although only a few of these are found with any significant frequency in human clinical isolates. *mcr-1* was the first transferrable colistin resistance gene identified (4) and is, by far, the most common (7). *mcr-1* has been identified in numerous environmental, animal-associated, and human-associated *Enterobacterales* isolates, with a worldwide distribution and with major foci in China, India, Southeast Asia, and Europe (1, 6, 10, 11). The frightening public heath potential of these genes, largely stemming from their transferrable nature, and an increasing realization of how truly widespread they are (10–12) have reinforced the necessity of rapid, reliable, and user-friendly methods for detecting colistin resistance in both the clinical and the public health laboratory settings (13).

Two new phenotypic methods to detect plasmid-mediated colistin resistance (PMCR) have been described: (i) the NG-Test MCR-1 (NG Biotech, Guipry, France) and (ii) the EDTA-colistin broth disk elution (EDTA-CBDE) method. The NG-Test MCR-1 is a single-use lateral flow immunoassay (LFA) for the rapid detection of the MCR-1 enzyme directly from bacterial colonies and was originally described by Volland et al. (14). The EDTA-CBDE method is a modification of the CBDE method, wherein a second set of tubes is set up to look for a reduction in MIC in the presence of EDTA via inhibition of MCR metalloenzyme-mediated resistance (15–17). We evaluated the performances of the NG-Test MCR-1 LFA (14) and EDTA-CBDE methods (18) for the detection of plasmid-borne, *mcr*-mediated colistin resistance across a spectrum of colistin-intermediate (MIC, $\leq$2 μg/ml) and colistin-resistant (MIC, $\geq$4 μg/ml) Gram-negative bacterial isolates. The isolates were collected as part of a larger Clinical and Laboratory Standards Institute (CLSI) multicenter study that evaluated the CBDE and colistin agar test (CAT) methods for determining colistin MICs (16).

MATERIALS AND METHODS

Bacterial isolates. A total of 238 isolates were tested at The Johns Hopkins Hospital (JHH), including 226 clinical isolates collected from 3 U.S. academic medical centers (JHH, The Mayo Clinic, and New York-Presbyterian/Columbia University Irving Medical Center) and 12 reference isolates from the Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) Antimicrobial Resistance Isolate Bank (AR Bank; www.cdc.gov/arisolatebank/), for which reference broth microdilution (rBMD) susceptibility results were determined as part of the larger CLSI study (16). The tested organisms included 68 *Klebsiella pneumoniae* isolates (including 36 resistant isolates and 2 isolates with *mcr-1*), 43 *Escherichia coli* isolates (including 15 resistant isolates, 6 isolates with *mcr-1*, and 1 isolate with *mcr-2*), 23 *Enterobacter cloacae* complex isolates (including 11 resistant isolates), 50 *Pseudomonas aeruginosa* isolates (including 5 resistant isolates), 50 *Acinetobacter* species isolates (including 27 resistant isolates), and 4 *mcr*-positive (for *mcr-1* [$n = 1$], *mcr-3* [$n = 2$], and *mcr-4* [$n = 1$]) *Salmonella* isolates. As the CBDE method was not endorsed by CLSI for use with *Acinetobacter* spp., the EDTA-CBDE method was evaluated only with *Enterobacterales* and *P. aeruginosa* isolates, whereas the NG-Test MCR-1 was evaluated with all isolates (16). Frozen stocks of isolates were subcultured onto 5% sheep blood agar (BA; Remel, Lenexa, KS) with a colistin disk placed in the first quadrant and incubated at 37°C for 16 to 24 h. A second subculture from the growth around the colistin disk, which was added to a new BA plate without a colistin disk, was performed prior to testing by both methods on the same day using the same subculture.

NG-Test MCR-1 LFA. The NG-Test MCR-1 LFA is a rapid single-use lateral flow immunoassay that utilizes streptavidin-labeled anti-MCR-1 mouse monoclonal antibodies to detect MCR-1 directly from bacterial colonies (14). The assay's nitrocellulose membrane has two regions: the test (T) region (T band), which contains the mouse anti-MCR-1 antibodies, and the control (C) region (C band), which contains goat anti-mouse monoclonal antibodies and which functions as an internal positive control. The formation of a visible red line for a positive sample is based on streptavidin-biotin-bovine serum albumin interaction in these regions.

The assay was performed according to the manufacturer's instructions provided in the package insert, with the exception of the inoculum (19), with each isolate being processed and tested in its own individual 1.5-ml tube and assay cartridge (Fig. 1). First, 5 drops (150 μl) of extraction buffer was added to each 1.5-ml tube. A heaped 1-μl loopful of fresh pure culture growth (the package insert recommends picking a single colony) was added to the 1.5-ml tube and briefly vortexed to create a homogenized suspension. Assay cassettes were opened and labeled immediately prior to use. Approximately 100 μl of

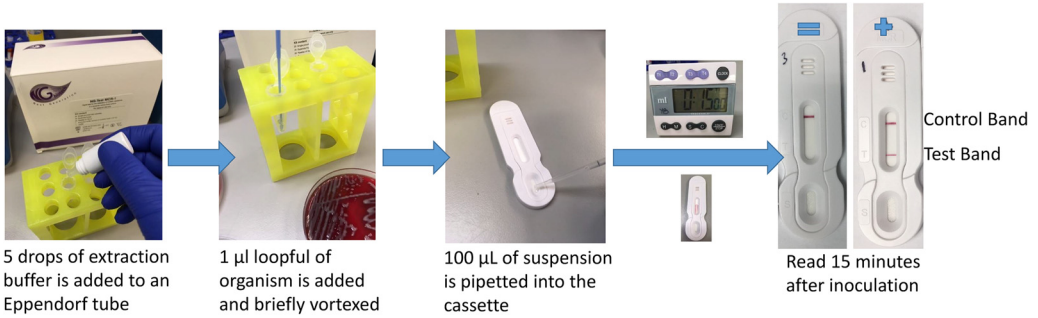

FIG 1 NG-Test MCR-1 lateral flow assay procedure.

the isolate-extraction buffer suspension was pipetted into the cassette sample well using manufacturer-provided single-use pipettes. LFAs were read 15 min after inoculation.

Interpretation of the results was based on the manufacturer's recommendations (19), with any visible T band being interpreted as positive for MCR-1, regardless of how strong or faint it was. All tests were required to have a positive C band in order to be considered valid. However, tests resulting in either an invalid (i.e., internal control failure) or a faint T band were repeated. Quality control was performed using *E. coli* 25922 as the negative control and an *mcr-1*-producing *E. coli* strain (AR Bank 0349) as the positive control.

EDTA-CBDE testing. CBDE and EDTA-CBDE testing were performed as previously described with the inclusion of an additional 0.4-μg/ml colistin dilution in this study (Fig. 2) (15–17). This was completed by setting up two sets of five cation-adjusted Mueller-Hinton broth (CA-MHB) glass tubes (Remel, Lenexa, KS): one set without EDTA added (CBDE) and one set with EDTA added (EDTA-CBDE). Each set included one tube containing 25 ml CA-MHB labeled 0.4 μg/ml and four tubes containing 10 ml CA-MHB labeled 1 μg/ml, 2 μg/ml, and 4 μg/ml and growth control (GC) for each isolate. EDTA was added to the second set of tubes, where 45 μl of 0.5 M EDTA was added to the 25-ml tube and 20 μl was added to each of the 10-ml CA-MHB tubes. Next, fixed numbers of colistin disks (10 μg; BD, Sparks, MD) were added to each set of tubes, as follows: 1 disk in the 25-ml CA-MHB tube and 1, 2, 4, and 0 disks in the remaining 10 ml CA-MHB tubes, yielding colistin concentrations of 0.4 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, and 0 μg/ml (growth control), respectively. The disk-impregnated CA-MHB tubes were incubated at room temperature for a minimum of 30 min to ensure sufficient elution of colistin from the disks. Fresh (18- to 24-h) isolate growth was suspended in 5 ml saline to yield a standardized 0.5 McFarland standard

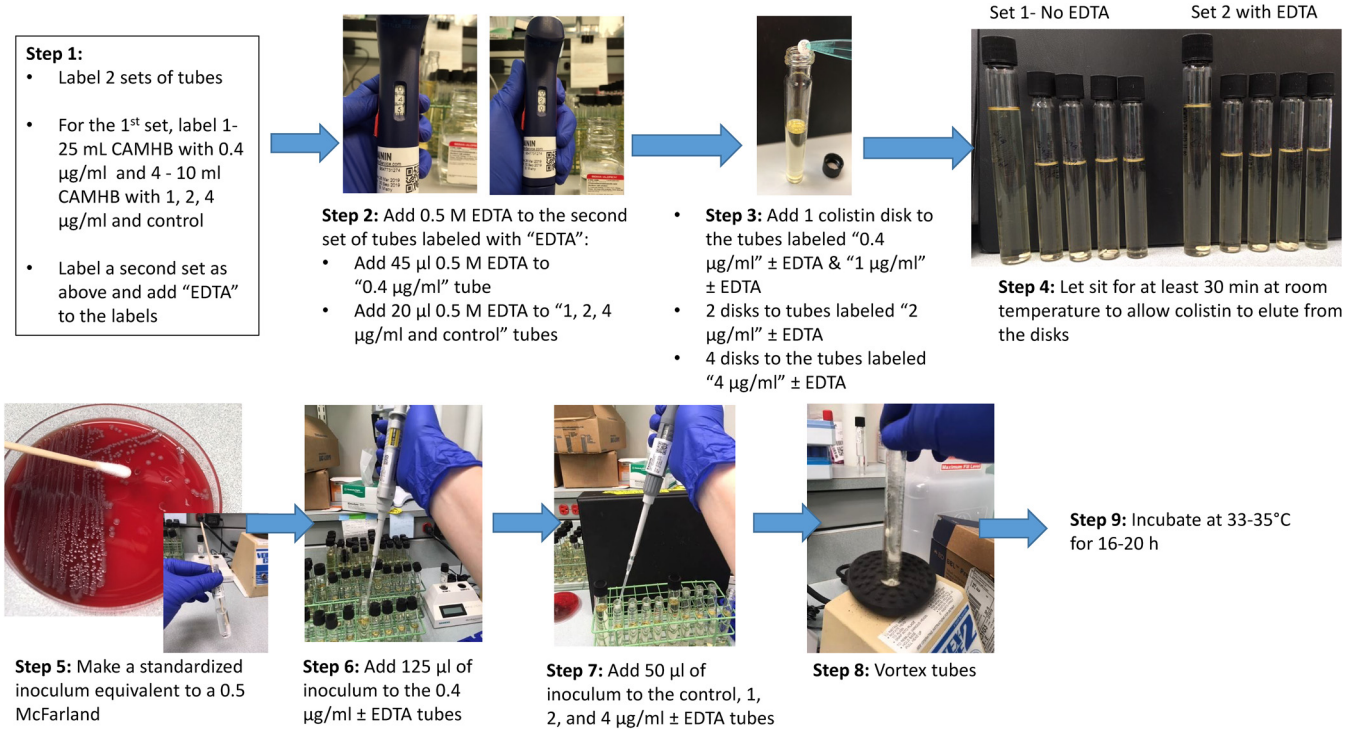

FIG 2 Step-by-step procedure for the EDTA-colistin broth disk elution (CBDE) method.

TABLE 1 Performance of NG-Test MCR-1 LFA versus expected result for isolates prior to repeat testing of isolates with faintly positive results

NG-Test MCR-1 LFA[a] result	No. of isolates with the following results by *mcr-1* and *mcr-2* PCR[b]:		
	Positive	Negative	Total
Positive	10	9[c]	19
Negative	0	219	219
Total	10	228	238

[a]LFA, lateral flow assay.
[b]The expected result was determined based on *mcr-1* and *mcr-2* PCR results.
[c]One *mcr-2*-producing *E. coli* isolate (AR Bank 0538) with a strong positive result and 8 isolates with faintly positive results that had negative results upon repeat testing.

inoculum, 50 μl of which was then inoculated into each 10-ml CA-MHB tube and 125 μl of which was inoculated into the 25-ml CA-MHB tube. A purity plate for each isolate was concurrently set up by streaking 10 μl of the original 0.5 McFarland standard solution onto BA (Remel, Lenexa, KS). The inoculated CA-MHB tubes were briefly vortexed. Tubes and purity plates were incubated at 33 to 35°C for 16 to 20 h. *P. aeruginosa* ATCC 27853 (expected results, a CBDE MIC of $\leq$1 μg/ml and a EDTA-CBDE MIC of $\leq$1 μg/ml indicate a negative result for PMCR) and *mcr-1*-producing *E. coli* strain AR Bank 0349 (expected results, a CBDE MIC of 2 to 4 μg/ml and a EDTA-CBDE MIC of $\leq$1 μg/ml indicate a positive result for PMCR) were used as controls.

The MIC was defined as the concentration in the tube with the lowest concentration of colistin with no visible growth (turbidity). Screening for PMCR by the EDTA-CBDE method was based on the detection of any reduction of the colistin MIC in the presence of EDTA compared to the MIC obtained by CBDE in the absence of EDTA (18). The EDTA-CBDE result was considered invalid when the CBDE result was read as 1 μg/ml and the EDTA-CBDE result was $\leq$0.4 μg/ml, as the MICs for the isolates were below the intermediate breakpoint ($\leq$2 μg/ml) and the isolates were interpreted to be negative for PMCR. The performance of the CBDE assay to determine colistin *in vitro* susceptibility testing results was not evaluated in this study, as results were previously reported for these isolates (16).

***mcr-1* and *mcr-2* PCR confirmatory testing.** All clinical isolates were tested for the *mcr-1* and *mcr-2* genes by conventional PCR, as previously described (20). *E. coli* AR Bank 0349 (an *mcr-1*-producing strain) and *E. coli* AR Bank 0538 (an *mcr-2*-producing strain) were used as positive controls, while *E. coli* 25922 was the negative control.

Repeat testing and data analysis. Tests were repeated when a discordant result was obtained for the NG-Test MCR-1 LFA and/or EDTA-CBDE methods, skipped tubes were observed for the EDTA-CBDE method, and/or an increase in MIC was observed in the presence of EDTA (21). If repeat testing resulted in resolution of the problem, then the repeat test result was accepted and included, with the original error being attributed to presumed random error. However, if repeat testing did not resolve the issue or if skipped tubes persisted, then the isolate and the result were subsequently excluded from the study.

Positive percent agreement (PPA) and negative percent agreement (NPA) were calculated based on the *mcr-1* and *mcr-2* PCR results for the clinical isolates or based on the expected results for the AR Bank isolates known to harbor an *mcr* gene. Isolates were presumed to be negative for PMCR by the EDTA-CBDE method if the colistin MICs were $\geq$4 μg/ml (resistant) and if the isolates were negative for *mcr-1* and *mcr-2* by PCR. For any colistin-resistant clinical isolate that was positive by the EDTA-CBDE method and negative by the *mcr-1* and *mcr-2* PCR, Illumina MiSeq sequencing (Illumina, San Diego, CA) and/or Nanopore (Oxford, England) whole-genome sequencing (WGS) results were used to evaluate the isolate for the presence of the *mcr-1* to *mcr-9* genes (22).

RESULTS

NG-Test MCR-1 LFA. Of the 238 isolates tested, the LFA was strongly positive for 11/238 (4.6%) isolates (known *mcr* positive) tested. An additional 8 isolates (8/238; 3.4%) were initially interpreted to be positive due to the presence of a faint line in the T-band region; these resolved to negative upon repeat testing. The PPA and NPA for MCR-1 detection prior to repeat testing were 100% and 96.1%, respectively (Table 1). Notably, the lone false-positive LFA result after repeat testing was due to a known MCR-2 producer (*E. coli* AR Bank 0538), which caused a strongly positive T band. The MCR-3- and MCR-4-producing *Salmonella* spp. were appropriately negative by the NG-Test MCR-1 LFA. The invalid rate due to improper wicking of the LFA was 1.3% (3/238), and the results for all tests were resolved upon repeat testing.

EDTA-CBDE. A total of 191 *Enterobacterales* and *P. aeruginosa* isolates were tested by the EDTA-CBDE method. Four *Enterobacterales* isolates were excluded, as their results were unresolved upon repeat testing, for an end total of 187 isolates being included in the analysis. The four isolates were removed due to repeat skipping of tubes

TABLE 2 Performance of EDTA-CBDE method for detecting PMCR

Result of EDTA-CBDE method	No. of isolates with the following results by combined reference method[a]:		
	Positive	Negative	Total
Positive	13	10[b]	23
Negative	0	164	164
Total	13	174	187

[a]The expected result was determined from a combination of antimicrobial susceptibility testing results determined by rBMD and *mcr-1* and *mcr-2* PCR results. Isolates were presumed to be negative for PMCR by the EDTA-CBDE method if the colistin MICs were ≥4 μg/ml (resistant) and the isolates were negative for *mcr-1* and *mcr-2* by PCR.
[b]Isolates with false-positive EDTA-CBDE results were confirmed to be negative for *mcr-1* to *mcr-9* genes by WGS.

(1 *Enterobacter cloacae* complex isolate and 1 *K. pneumoniae* isolate) and 2 odd results, where the MIC increased in the presence of EDTA (1 *Enterobacter cloacae* complex isolate and 1 *K. pneumoniae* isolate). EDTA-CBDE had an overall PPA and NPA of 100% and 94.3%, respectively (Table 2). PPA was 100% for both *Enterobacterales* and *Pseudomonas aeruginosa*; however, EDTA-CBDE had a slightly lower NPA of 94.2% with *Enterobacterales*, whereas it had an NPA of 96.0% with *P. aeruginosa*.

There were a total of 10 false-positive results for the EDTA-CBDE method, but the results were confirmed upon repeat testing, in which the isolates were negative for the *mcr-1* through *mcr-9* genes based on WGS results. The isolates with false-positive results included 4 *E. coli* isolates, 3 *K. pneumoniae* isolates, 1 *E. cloacae* complex isolate, 2 *P. aeruginosa* isolates (Table 3). Notably, the 4 *E. coli* isolates with false-positive results by EDTA-CBDE accounted for 50% (4/8) of all colistin-resistant *E. coli* isolates included in this study.

Nine isolates had a CBDE MIC of 1 μg/ml with an EDTA-CBDE result of ≤0.4 μg/ml (2 *E. coli* isolates, 2 *K. pneumoniae* isolates, 1 *E. cloacae* complex isolate, 4 *P. aeruginosa* isolates), rendering the corresponding EDTA-CBDE results invalid, as they fell below the intermediate breakpoint, and the isolates were interpreted to be negative for PMCR. Due to the new/revised CLSI breakpoints being set at 2 μg/ml for intermediate and 4 μg/ml for resistant, the 0.4-μg/ml dilution is not necessary when testing at the breakpoints for the CBDE and EDTA-CBDE methods (16).

***mcr-1* and *mcr-2* PCR.** All clinical isolates underwent confirmatory testing by PCR for the presence or absence of *mcr-1* and *mcr-2*. Only a single isolate out of the 226 total clinical isolates (1/226; 0.4%) tested by PCR was positive for *mcr-1*; there were no clinical isolates positive for *mcr-2* by PCR. The single *mcr-1*-positive clinical isolate was also positive by the NG-Test MCR-1 LFA and the EDTA-CBDE methods.

TABLE 3 Clinical isolates associated with false-positive EDTA-CBDE results[a]

Study identifier	Organism	MIC (μg/ml)			ΔMIC (no. of DD)
		rBMD[b]	CBDE	EDTA-CBDE	
CRE 174	*Escherichia coli*	8	>4	1	≥2
421	*Escherichia coli*	8	4	≤0.4	≥3
E. coli #23 Mayo	*Escherichia coli*	8	>4	4	≥1
E. coli #65 Mayo	*Escherichia coli*	8	4	≤0.4	≥2
CRE 44	*Klebsiella pneumoniae*	≤0.25	2	1	≥1
CRE 196	*Klebsiella pneumoniae*	>16	>4	4	≥1
CRE 529	*Klebsiella pneumoniae*	>16	>4	4	≥1
NR1677 CUMC	*Enterobacter cloacae* complex	>16	>4	≤0.4	≥3
P. aeruginosa #2 JHMI	*Pseudomonas aeruginosa*	16	>4	≤0.4	≥3
IHMA-517613	*Pseudomonas aeruginosa*	>16	>4	2	≥2

[a]rBMD, reference broth microdilution; CBDE, colistin broth disk elution; EDTA-CBDE, EDTA-colistin broth disk elution; ΔMIC, difference in MICs between the CBDE and EDTA-CBDE methods; DD, doubling dilutions. Samples with false-positive EDTA-CBDE results were confirmed to be negative for the *mcr-1* to *mcr-9* genes by WGS.
[b]The reference MIC was calculated by taking the average of two rBMD results, rounded up to the nearest 2-fold ($\log_2$) dilution MIC (16).

DISCUSSION

The chronic and systematic use and misuse of antimicrobials have made multidrug-resistant bacterial infections an increasing reality for both clinicians and public health authorities. As resistance continues to become increasingly prevalent, clinical microbiology laboratories will be required to provide reliable and rapid information about a particular isolate's susceptibility to new, rarely used, and/or last-line antimicrobial agents (23). Information about antimicrobial resistance phenotypes and their respective mechanism(s) also has significant value for public health and hospital infection control epidemiologists as they track the local, regional, and global spread of resistance, especially that conferred by plasmid-borne resistance genes, which are readily transferrable. The NG-Test MCR-1 LFA and the EDTA-CBDE methods are two recently described assays that can be used to screen Gram-negative bacterial isolates for PMCR.

In this study, we demonstrate that the NG-Test MCR-1 LFA provides rapid (15 min for the time to a result), sensitive (100%), and specific (99%) screening for the presence of MCR-1 in American bacterial isolates directly from culture. These findings are largely consistent with those described by Volland et al. (14), who helped develop the assay and who first described the performance characteristics of the assay using European isolates. They observed a sensitivity of 100% and a specificity of 98%. In contrast, we experienced a relatively large number of faintly positive T bands (i.e., false-positive results; 3.4%), resulting in an initially decreased specificity that was resolved upon repeat testing. However, it is important to note that the package insert specifically instructs the reader to consider any T band to be a positive result, regardless of intensity (19). While Volland et al. (14) did note some faintly positive results, they specifically noted that these, in fact, either were truly positive for MCR-1 or were due to cross-reactivity with MCR-2.

One possible explanation for this difference may be rooted in the fact that our inoculum volume was larger than that of Volland et al. (14). The decision to err on the side of a larger inoculum was made because we observed a faintly positive T band when testing our MCR-1-positive control isolate during the initial preexperiment walk-through using the recommended inoculum volume of a single colony (19). We therefore opted to use a heaped 1-μl loopful as the inoculum to help ensure strongly positive T bands, which may partially explain the increased numbers of faintly positive T bands that we observed, although this would not explain why these discrepant results resolved upon repeat testing with the same inoculum volume. Another, more likely explanation for our increased number of initial false-positive T bands is that there is an inherent degree of subjectivity in interpreting the test bands, particularly in cases with faintly positive T bands.

The LFA's cross-reactivity with MCR-2 (14, 19) is a dual-edged sword, providing additional (albeit off-label) detection coverage of additional MCR enzymes while simultaneously reducing the assay's analytical specificity, as defined by the manufacturer. The possibility of cross-reactivity with some MCR-2 variants is noted in the NG-Test MCR-1 LFA package insert (19). No cross-reactivity with MCR-3 and MCR-4 producers was observed in this study. One major disadvantage of the LFA is that it is (nominally) MCR-1 specific, failing to detect other members of the MCR family. This potentially limits the utility of negative results in the absence of additional testing for phenotypic resistance, particularly in low-incidence settings. The necessity of taking epidemiological factors into account is illustrated by the fact that we were able to find only a single MCR-1-positive clinical isolate for our study, despite specifically evaluating many colistin-resistant isolates from 3 major U.S. academic medical centers.

The EDTA-CBDE method also provided a sensitive (100%) and specific (94.3%) screening for the presence of PMCR among *Enterobacterales* and *P. aeruginosa* isolates directly from culture, albeit with a longer time to a result than the LFA (16 to 20 h versus 15 to 20 min). However, its turnaround time (TAT) is comparable to that of other MIC-based methods that simultaneously determine *in vitro* antimicrobial susceptibility results and that detect PMCR by determining polymyxin MICs in the presence and

TABLE 4 Summary of advantages and disadvantages of using the NG-Test MCR-1 LFA and EDTA-CBDE methods to screen for plasmid-mediated colistin resistance compared to PCR

Test	Advantages	Disadvantages
NG-Test MCR-1 LFA	Setup requires minimal hands-on time (approximately 1 min per isolate)	Detects only MCR-1-mediated colistin resistance
	Rapid results (15 min)	Limited-to-no clinical utility; result significance is largely restricted to epidemiological, surveillance, infection control, and research purposes
	Simple, easy-to-perform procedure	Subjective interpretation of faint lines may cause false-positive results
	Reliable performance regardless of the Gram-negative bacterium tested	New test, not yet cleared for use in the United States
	Limited ability to also detect MCR-2 through cross-reactivity	May be expensive relative to other phenotypic tests
EDTA-CBDE	Combined with CBDE, so testing provides clinically actionable results (MIC values)	Not endorsed for use with *Acinetobacter* isolates
	Reliable for *Enterobacterales* species and *Pseudomonas aeruginosa*	Materials required for testing may be more expensive than those required for other phenotypic tests and may be perishable
	Demonstrated to detect *mcr-1* through *mcr-4*	Setup requires 10–20 min of hands-on time per isolate, depending on the test volume, which can be a potential source of variability
		Results take 16–20 h
		Subjective interpretation of growth in broth, subject to potential variability between readers and across isolates
		This is a screening test; EDTA is not a specific inhibitor of *mcr* and may cross-react with other non-plasmid-mediated resistance mechanisms; therefore, confirmatory testing (i.e., by *mcr* PCR) is required for positive results
mcr PCR	Accurate and reliable	Expensive
	Users can customize primers or multiplex assays to target multiple genes across entire *mcr* family	Requires a highly skilled technologist, and setup requires significant hands-on time
		Testing is largely limited to reference laboratories
		Mutations in target gene may cause false-negative results
		Utility limited to epidemiological, surveillance, infection control, and research purposes

absence of a chelator (24, 25). As previously noted, EDTA is not a specific inhibitor of *mcr*, with its inclusion resulting in a slightly decreased specificity of the assay, which is the reason why EDTA-CBDE is a screening test for which all positive results must be confirmed by a secondary method (e.g., a molecular method) (15). Furthermore, the EDTA-CBDE method is relatively easy to perform using materials that are readily available in most clinical laboratories and is capable of detecting multiple *mcr* variants (e.g., *mcr-1* to *mcr-4* were detected in this study).

Unfortunately, the CBDE and EDTA-CBDE methods are not recommended for use with *Acinetobacter* species due to high error rates, and large numbers of isolates are excluded from analysis due to the absence of a reference MIC (16). Recently, a resazurin-based rapid test to detect colistin resistance in *Acinetobacter* isolates was described and had a 93.3% sensitivity and specificity (26). This method does not distinguish between chromosome- or plasmid-mediated colistin resistance but provides an alternative to BMD to detect colistin resistance among *Acinetobacter* species.

There are some limitations to the EDTA-CBDE method. First, the materials may be costly relative to the cost of the materials used for other phenotypic methods, but the costs are comparable to those of the NG-Test MCR-1 LFA (estimated cost, $15 to $20). Clinical laboratories offering this testing need to have available stores of perishable, premade 10-ml CA-MHB tubes, which can cost anywhere from $1 to $5 per tube, with each tested isolate requiring 8 tubes for the combined CBDE and EDTA-CBDE methods. This excludes the need for 25-ml CA-MHB tubes, as the newly CLSI-approved colistin

breakpoints were set at 2 μg/ml for intermediate and 4 μg/ml for resistant, thereby eliminating the need for testing at a concentration of 0.4 μg/ml. Alternatively, labs may choose to prepare and dispense their own 10-ml CA-MHB tubes to reduce costs. Furthermore, interpretation of broth turbidity can be subjective and subject to variability both between technologists and between bacterial isolates.

While both tests have their own individual advantages and disadvantages, there is some overlap between the NG-Test MCR-1 LFA and EDTA-CBDE in both categories (Table 4). For example, both methods are sensitive for detecting MCR-1-mediated colistin resistance. On the other hand, because EDTA-CBDE is a screening test, all positive results require subsequent confirmatory testing by a second method (e.g., *mcr* PCR). Additionally, neither the NG-Test MCR-1 LFA nor EDTA-CBDE provides clinically actionable results as a stand-alone test. However, the concurrent CBDE testing required for EDTA-CBDE interpretation would ultimately result in an actionable colistin MIC value, as was previously described (17). Importantly, this means that for clinical microbiology laboratories, the utility of both the NG-Test MCR-1 LFA and the EDTA-CBDE test is largely centered around epidemiological, surveillance, hospital infection control, and research purposes, as different methods (e.g., CBDE, CAT, or rBMD) will be used to determine colistin *in vitro* susceptibility results.

Limitations of this study include the fact (i) testing was performed at a single center, (ii) only a single manufacturer of CA-MHB and colistin disks was used to evaluate the EDTA-CBDE method, and (iii) few *mcr*-positive isolates were evaluated, in spite of assessing as many colistin-resistant isolates as possible from 3 U.S. academic medical centers.

Despite the rarity of PMCR among clinical isolates in the United States, these methods are valuable tools that may be implemented in public health and clinical microbiology laboratories to further discern the mechanism of resistance among colistin-resistant Gram-negative isolates and to screen for PMCR for infection prevention and control purposes.

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REFERENCES

1. Butaye P, Wang C. 2018. Colistin resistance, beyond the current knowledge. EBioMedicine 34:16–17. https://doi.org/10.1016/j.ebiom.2018.08.002.
2. Jerke KH, Lee MJ, Humphries RM. 2016. Polymyxin susceptibility testing: a cold case reopened. Clin Microbiol Newsl 38:69–77. https://doi.org/10.1016/j.clinmicnews.2016.04.003.
3. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev 30:557–596. https://doi.org/10.1128/CMR.00064-16.
4. 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.
5. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmann M. 2019. Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant, colistin-susceptible Salmonella enterica serotype Typhimurium isolate using a combination of high-throughput, in silico screening and functional analysis. bioRxiv https://doi.org/10.1101/539361.
6. 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.
7. Wise MG, Estabrook MA, Sahm DF, Stone GG, Kazmierczak KM. 2018. Prevalence of mcr-type genes among colistin-resistant Enterobacteriaceae collected in 2014–2016 as part of the INFORM global surveillance program. PLoS One 13:e0195281. https://doi.org/10.1371/journal.pone.0195281.
8. Ezadi F, Ardebili A, Mirnejad R. 2018. Antimicrobial susceptibility testing for polymyxins: challenges, issues, and recommendations. J Clin Microbiol 57:e01390-18. https://doi.org/10.1128/JCM.01390-18.
9. Wang X, Wang Y, Zhou Y, Wang Z, Wang Y, Zhang S, Shen Z. 2019. Emergence of colistin resistance gene mcr-8 and its variant in Raoultella ornithinolytica. Front Microbiol 10:228. https://doi.org/10.3389/fmicb.2019.00228.
10. Wang R, Van Dorp L, Shaw LP, Bradley P, Wang Q, Wang X, Jin L, Zhang Q, Liu Y, Rieux A, Dorai-Schneiders T, Weinert LA, Iqbal Z, Didelot X, Wang H, Balloux F. 2018. The global distribution and spread of the mobilized colistin resistance gene mcr-1. Nat Commun 9:1179. https://doi.org/10.1038/s41467-018-03205-z.
11. Mulvey MR, Mataseje LF, Robertson J, Nash JHE, Boerlin P, Toye B, Irwin R, Melano RG. 2016. Dissemination of the mcr-1 colistin resistance gene. Lancet Infect Dis 16:289–290. https://doi.org/10.1016/S1473-3099(16)00067-0.
12. Castanheira M, Griffin MA, Deshpande LM, Mendes RE, Jones RN, Flamm RK. 2016. Detection of mcr-1 among Escherichia coli clinical isolates collected worldwide as part of the SENTRY Antimicrobial Surveillance Program in 2014 and 2015. Antimicrob Agents Chemother 60:5623–5624. https://doi.org/10.1128/AAC.01267-16.
13. Giske CG, Kahlmeter G. 2018. Colistin antimicrobial susceptibility test-

ing—can the slow and challenging be replaced by the rapid and convenient? Clin Microbiol Infect 24:93–94. https://doi.org/10.1016/j.cmi.2017.10.007.
14. Volland H, Dortet L, Bernabeu S, Boutal H, Haenni M, Madec JY, Robin F, Beyrouthy R, Naas T, Simon S. 2019. Development and multicentric validation of a lateral flow immunoassay for rapid detection of MCR-1-producing Enterobacteriaceae. J Clin Microbiol 57:e01454-18. https://doi.org/10.1128/JCM.01454-18.
15. Bell DY, Bergman Y, Kazmi A, Lewis S, Tamma PD, Simner PJ. 2018. A novel phenotypic method to screen for plasmid-mediated colistin resistance, poster Friday-CPHM LB3. Abstr ASM Microbe 2018, Atlanta, GA. American Society for Microbiology, Washington, DC. http://pathology.jhu.edu/department/training/symposium/posters/2018/poster-13.pdf.
16. Humphries RM, Green DA, Schuetz AN, Bergman Y, Lewis S, Yee R, Stump S, Lopez M, Macesic N, Uhlemann A-C, Kohner P, Cole N, Simner PJ. 2019. Multicenter evaluation of colistin broth disk elution and colistin agar test: a report from the Clinical and Laboratory Standards Institute. J Clin Microbiol 57:e01269-19. https://doi.org/10.1128/JCM.01269-19.
17. 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.
18. Bell DT, Bergman Y, Kazmi AQ, Lewis S, Tamma PD, Simner PJ. 2019. A novel phenotypic method to screen for plasmid-mediated colistin resistance among Enterobacteriales. J Clin Microbiol 57:e00040-19. https://doi.org/10.1128/JCM.00040-19.
19. NG Biotech. 2017. NG-Test MCR-1 package insert. NG Biotech, Guipry, France.
20. 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(27):pii=30280. https://doi.org/10.2807/1560-7917.ES.2016.21.27.30280.
21. Clinical and Laboratory Standards Institute. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Clinical and Laboratory Standards Institute, Wayne, PA.
22. 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.
23. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis 40:1333–1341. https://doi.org/10.1086/429323.
24. 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.
25. 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.
26. Germ J, Poirel L, Kisek TC, Spik VC, Seme K, Premru MM, Zupanc TL, Nordmann P, Pirs M. 2019. Evaluation of resazurin-based rapid test to detect colistin resistance in Acinetobacter baumannii isolates. Eur J Clin Microbiol Infect Dis 38:2159–2162. https://doi.org/10.1007/s10096-019-03657-1.