

## Evaluation of a Screening Method for the Detection of Colistin-Resistant *Enterobacteriaceae* in Stool

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Emergence of mobile colistin resistance (*mcr*)-containing *Enterobacteriaceae* is a public health threat, prompting enhanced surveillance through the Centers for Disease Control and Prevention. We evaluated a selective culture medium for the isolation of *Enterobacteriaceae* with non-wild-type colistin minimum inhibitory concentrations, including those with *mcr-1* genes, in spiked stool samples.

**Keywords.** colistin resistance; *mcr* *Enterobacteriaceae*; screening.

Mobile colistin resistance (*mcr*) genes encode resistance to polymyxins, a class of antibiotics used when treatment options are limited. The *mcr* genes are plasmid-mediated, which may facilitate their transfer between bacteria, resulting in the spread of resistance. Recently, there has been an increase in the isolation of bacteria harboring *mcr* genes in humans and animals in the United States, prompting the US Centers for Disease Control and Prevention (CDC) to enhance surveillance for organisms with these genes [1].

The ability to screen clinical samples for bacteria displaying non-wild-type colistin minimum inhibitory concentrations (MICs) due to carriage of *mcr* genes would improve our understanding of this problem. Zurfluh et al. [2] reported fecal carriage rates of colistin-resistant *Enterobacteriaceae*,

including *mcr*-containing isolates, using a selective culture medium containing colistin, vancomycin, and amphotericin B [2]. We evaluated this screening method for the detection of *Enterobacteriaceae* displaying non-wild-type colistin MICs in stool spiked with well-characterized isolates containing the *mcr-1* gene.

### METHODS

The screening medium was made using Luria-Bertani (LB) medium. In an Erlenmeyer flask, 25 g of LB and 15 g of Bacto agar powder were dissolved in 1000 mL of double distilled water (ddH<sub>2</sub>O). The solution was autoclaved on a liquid cycle at 121°C for 15 minutes and cooled in a water bath to 56°C. Once it cooled, 1 mL of colistin sulfate (stock solution 4000 mg/dL in ddH<sub>2</sub>O; Sigma-Aldrich, St. Louis, MO), 1 mL of vancomycin HCl (stock solution 10 000 mg/mL in ddH<sub>2</sub>O; Sigma-Aldrich, St. Louis, MO), and 1 mL of amphotericin B (5000 mg/mL in ddH<sub>2</sub>O; Sigma-Aldrich, St. Louis, MO) were added, for final concentrations of 4, 10, and 5 mg/mL.

Nine members of the family *Enterobacteriaceae* were used from panels obtained from the CDC and US Food and Drug Administration (FDA) Antimicrobial Resistance Isolate Bank (AR Isolate Bank; <https://www.cdc.gov/drugresistance/resistance-bank/index.html>). The presence of resistance mechanisms was established through isolate whole-genome sequencing using the ResFinder database (last updated June 2, 2016, and accessed on October 25, 2016) [3]. Seven of the isolates carried *mcr-1* genes. The mechanism of colistin resistance for the remaining isolates was unknown. MICs for colistin were determined via broth microdilution by the AR Isolate Bank (Table 1). Using frozen stock (−80°C), each isolate was subcultured twice on tryptic soy agar with 5% sheep's blood (Trypticase soy agar with 5% sheep's blood [TSA with 5% SB]; Becton, Dickinson and Company, Sparks, MD) and incubated in ambient air at 35°C ± 2°C for 18 to 24 hours before use.

Donated fecal matter capsules were used for the spiked stool samples. Donor feces were screened for stool pathogens and multidrug-resistant bacteria and packaged into capsules, as described by Youngster et al. [4]. Each capsule was kept frozen (−80°C) until use. Once each capsule was removed from the freezer, the outer capsule was manually removed, leaving the inner capsule in place. Capsules were placed in 2 mL of sterile water and mixed on a rotator until thawed.

To determine the limit of detection (LOD) of the medium for *Enterobacteriaceae* with a colistin MIC ≥ 4, serial 10-fold dilutions of an inoculum with an optical density of 0.5 McFarland (approximately 10<sup>8</sup> colony-forming unit [CFU]/mL) of AR Isolate Bank *Escherichia coli* 0346 and *Klebsiella pneumoniae* 0125 were

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**Table 1. Isolate Detection for LB Medium With Colistin, Vancomycin, Amphotericin B in Stool Spiked With *Enterobacteriaceae* With Elevated MICs to Colistin**

AR Isolate Bank No.	Species	Colistin MIC, µg/mL <sup>a</sup>	Mechanism of Colistin Resistance	Isolate Detected at 10 <sup>2</sup> and 10 <sup>3</sup> CFU/mL
AR-Bank 0346	<i>E. coli</i>	4/>4	<i>mcr-1</i>	Y
AR-Bank 0349	<i>E. coli</i>	2–4/2	<i>mcr-1</i>	Y
AR-Bank 0350	<i>E. coli</i>	4/4	<i>mcr-1</i>	Y
AR-Bank 0493	<i>E. coli</i>	8/>4	<i>mcr-1</i>	Y
AR-Bank 0494	<i>E. coli</i>	8/>4	<i>mcr-1</i>	Y
AR-Bank 0495	<i>E. coli</i>	4/4	<i>mcr-1</i>	Y
AR-Bank 0496	<i>Salmonella enteritidis</i>	8/>4	<i>mcr-1</i>	Y
AR-Bank 0040	<i>K. pneumoniae</i>	4/>4	Unknown	Y
AR-Bank 0125	<i>K. pneumoniae</i>	>4/4	Unknown	Y

Abbreviations: CDC, Centers for Disease Control and Prevention; CFU, colony-forming unit; CLSI, Clinical and Laboratory Standards Institute; FDA, Food and Drug Administration; LB, Luria-Bertani; MIC, minimum inhibitory concentration.

<sup>a</sup>MIC performed by the CDC and FDA Antimicrobial Resistance Isolate Bank/MIC performed via broth microdilution per CLSI guidelines on isolate identified in stool.

prepared; 100 µL of each dilution was pipetted into 900 µL of thawed donated fecal matter, and 0.5 mL of each spiked stool was placed in 4.5 mL of an *Enterobacteriaceae* enrichment broth (EE broth; Hardy Diagnostics, Santa Maria, CA) and incubated at 35°C ± 2°C [2]. After 24 hours of incubation, 10 µL of the spiked EE broth was inoculated onto the selective medium and incubated in ambient air at 35°C ± 2°C for 48 hours. 0.5 mL of nonspiked thawed donor fecal matter was placed in 4.5 mL of EE broth, incubated, and subcultured on the selective medium as a negative control. All unique colonies were subcultured to TSA with 5% SB and MacConkey II agar (MAC; Becton, Dickinson and Company, Sparks, MD) and were identified using the Vitek MS system, version 2.0, in vitro diagnostic database (bioMérieux, Durham, NC). MICs to colistin, amikacin, gentamicin, tobramycin, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, levofloxacin, ertapenem, imipenem, meropenem, and trimethoprim-sulfamethoxazole were determined for all isolates. Broth microdilution was performed using the Sensititre Vizion system (ThermoFisher Scientific, Waltham, MA) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [5]. Results were compared with MICs reported for these drugs by the AR Isolate Bank for accurate identification of the spiked organisms [5].

To determine the sensitivity of the screening medium for detection of *Enterobacteriaceae* with a colistin MIC ≥4, thawed donated fecal matter was spiked with 1 of the 9 isolates and serially diluted to final concentrations of 10<sup>2</sup> or 10<sup>3</sup> CFU/mL (Table 1). Each spiked stool was processed as described above, and nonspiked thawed donor fecal matter was used as a negative control. All unique colonies, subcultured to TSB with 5% SB and MAC, underwent organism identification and susceptibility testing for accurate identification of all organisms.

## RESULTS

AR Isolate Bank *E. coli* 0346 and *K. pneumoniae* 0125 were successfully isolated at concentrations of 10<sup>2</sup> CFU/mL and above

on all of the selective medium after 48 hours of incubation. Other organisms identified included *Serratia* and *Hafnia* spp., which have intrinsic resistance to colistin, and *Enterobacter* spp. and *Clostridium paraputrificum*, both of which have the potential for non-wild-type colistin MICs [2, 5–7]. No *E. coli* or *K. pneumoniae* were isolated from the nonspiked thawed donated fecal matter sample.

Using an LOD of 10<sup>2</sup> CFU/mL, donated fecal matter was then spiked with 1 of the 9 isolates at a concentration of 10<sup>2</sup> and 10<sup>3</sup> CFU/mL and cultured using the methods described above. All 9 organisms were isolated from the selective medium after 48 hours of incubation at concentrations of 10<sup>2</sup> and 10<sup>3</sup> CFU/mL (Table 1). *Serratia fonticola* was also isolated. No strains of *E. coli*, *K. pneumoniae*, or *Salmonella enteritidis* were isolated from the medium inoculated with the nonspiked thawed donated fecal matter sample. The sensitivity of this screening medium was 100%.

## DISCUSSION

The emergence of plasmid-mediated resistance to colistin in *Enterobacteriaceae* through acquisition of *mcr* genes is a public health concern. Screening algorithms to identify clinical isolates that may carry these resistance genes are needed. Our results show that the selective culture method described by Zurfluh et al. [2] can detect *Enterobacteriaceae* with non-wild-type colistin MICs, including those with known *mcr* genes, at low concentrations in spiked stool obtained from healthy, asymptomatic adults.

No large studies exist evaluating the concentration of *mcr*-bearing bacteria in the stool of colonized patients, and the limited data examining this have conflicting results. Dona et al. identified *mcr-1*-bearing *E. coli* in the stool of 2 asymptomatic subjects at approximately the LOD of a real-time polymerase chain reaction (PCR) assay for the detection of *mcr-1*-bearing bacteria (10 g DNA copies/reaction), indicating that concentrations of *mcr-1*-bearing *Enterobacteriaceae* in the stool of colonized patients

could be quite low [8]. Conversely, Nijhuis et al. reported the identification of an *mcr-1*-bearing *E. coli* in a surveillance stool specimen well below the cycle threshold for the LOD of a real-time PCR assay (3–30 CFU/reaction), indicating the presence of this organism at a fairly high concentration [9]. If we examine data regarding the concentration of other bacteria known to colonize the gastrointestinal track of humans, such as vancomycin-resistant *Enterococci* (VRE), concentrations of this organism in the stool of colonized patients range from  $10^{2.5}$  to  $10^{8.1}$  CFU/mL [10]. Extrapolating this information to *mcr*-bearing bacteria in the stool of colonized patients, this screening method could reliably detect these organisms.

Our study has limitations. First, only 9 isolates were used for the evaluation of this screening medium and method. This number was based on the availability of well-characterized strains from the AR Isolate Bank. Many MICs to colistin for isolates containing other *mcr* genes have been reported to be high enough to overcome the concentration of colistin in this medium; a formal evaluation of isolates with other *mcr* genes should be performed [1, 11–14]. Second, this screening method is capable of isolating *Enterobacteriaceae* with non-wild-type colistin MICs, but it does not distinguish between *mcr*- and non-*mcr*-mediated colistin resistance mechanisms. Confirmatory methods to detect the presence of *mcr* genes, such as PCR, are required; this can be done through the CDC's AR Lab Network [15]. Third, the specificity of this medium for the detection of non-wild-type colistin MICs was not specifically addressed. However, the growth of nonspiked *Enterobacteriaceae* was limited to those with intrinsic resistance or the potential for non-wild-type phenotypes to colistin. Despite this, formal evaluation is needed. Finally, the screening method was evaluated using spiked stool from asymptomatic adults; further study, particularly in subjects who are potential carriers of *Enterobacteriaceae* with non-wild-type colistin MICs, is needed.

In conclusion, the selective culture method of Zurfluh et al. [2] is valid for detecting *Enterobacteriaceae* with non-wild-type phenotypes to colistin, including those with known *mcr-1* mutations in human stool. The ease of the screening method makes it an appropriate initial screening tool for most clinical laboratories.

## Notes

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