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RESEARCH ARTICLE

A Novel Lipid-Based MALDI-TOF Assay for the Rapid Detection of Colistin-Resistant *Enterobacter* Species

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ABSTRACT *Enterobacter* species are classified as high-priority pathogens due to high prevalence of multidrug resistance from persistent antibiotic use. For *Enterobacter* infections caused by multidrug-resistant isolates, colistin (polymyxin E), a last-resort antibiotic, is a potential treatment option. Treatment with colistin has been shown to lead to emergence of polymyxin resistance. The primary mechanism for colistin resistance is modification of terminal phosphate moieties of lipid A, leading to decreased membrane electronegativity and reducing colistin binding affinity. Detection of these modifications, including the addition of phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (Ara4N), can be used for prediction of colistin resistance using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The objective of this study was to identify lipid A markers for colistin resistance in *Enterobacter* species and *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*). Using a collection of *Enterobacter* and *Klebsiella aerogenes* clinical isolates, broth MICs for colistin were determined initially. Subsequently, killing assays were carried out to determine how the concentration of colistin at which there is approximately 50% survival ($kill_{50}$) equates to their MICs. Finally, lipid A analysis was conducted via MALDI-TOF MS using the novel rapid extraction method, termed fast lipid analysis technique (FLAT), to correlate MIC and killing efficacy with predictive lipid A modifications. Sensitivity and specificity of the MS assay compared to MIC interpretation were 100% and 53.4%, respectively. A receiver operator characteristic (ROC) demonstrated that MS was highly correlated with killing, with area under the curve of 0.97. This analysis demonstrated the potential utility of MALDI-TOF MS as a rapid diagnostic platform of colistin resistance in *Enterobacter* species.

IMPORTANCE In this study, we develop a novel method for identifying colistin resistance in *Enterobacter* species and *Klebsiella aerogenes* without performing antimicrobial susceptibility testing. Typically, susceptibility testing requires an additional 24 to 48 h, while the MS assay described in this study allows for resistant identifications in under 1 h after initial culture. Identification using MALDI-TOF MS would save time and prevent inappropriate use of colistin. MALDI-TOF MS is an easy-to-use, readily available, robust diagnostic tool in clinical laboratories. Furthermore, this study highlights limitations of polymyxin susceptibility testing. Use of a killing assay best captures how colistin treats infection and is shown to be highly correlated with our MS assay; thus, the MS assay in this study effectively predicts how colistin would treat a patient's infection. Use of MALDI-TOF MS for accurate and early identification of antimicrobial resistance can improve antimicrobial stewardship and patient outcomes.

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Enterobacter is a genus of Gram-negative rod-shaped bacteria and part of the *Enterobacteriaceae* family (1). A common health care-associated pathogen, *Enterobacter* species can cause infections in the respiratory tract, urinary tract, and surgical wounds (2). Additionally, *Enterobacter* species are a common cause of hospital-acquired bacteremia. *Enterobacter* species are included in the list of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, common multidrug-resistant organisms that are considered highest priority for developing new antimicrobial therapies due to increased levels of resistance, virulence, and lack of available therapies by both the CDC and WHO (3, 4). Colistin is one of the few active antibiotics for treatment of multidrug-resistant (MDR) *Enterobacter* infections (5). However, the emergence and increasing incidence of colistin resistance have led to further clinical complications in treating infections due to a reduced number of viable treatment options (5).

Colistin, or polymyxin E, is a cationic lipopeptide and "last-resort" antibiotic used for MDR Gram-negative infections, such as those producing extended-spectrum beta-lactamases and those resistant to carbapenems (6), which is an increasing concern in clinical isolates of *Enterobacter* species (7). Targeting the cell membrane, cationic colistin binds with anionic lipopolysaccharide (LPS) through electrostatic interactions (8–11). Colistin resistance is conferred by reduction of the electronegativity of the cell membrane through modification of the terminal phosphate residues of lipid A, the membrane anchor of LPS, thereby decreasing the binding affinity of colistin (12). Lipid A modifications that reduce electronegativity of the cell membrane include the addition of phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (Ara4N), which have been detailed in longitudinal studies for colistin-resistant *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (13–17). With increasing usage of colistin, increasing levels of resistance of *P. aeruginosa* have been observed, especially in patients with cystic fibrosis (18). The growing prevalence of colistin-resistant pathogens has created a significant demand for the development of a rapid diagnostic to differentiate resistant isolates from intermediate or sensitive isolates.

Currently, phenotypic antibiotic susceptibility testing (AST) is the standard method for identification of resistance in pathogens and is performed via broth microdilution or disc diffusion assay to determine the MIC. The MIC is the lowest concentration of antibiotic where growth inhibition is observed. Testing for colistin susceptibility using these classic methods is problematic (19, 20). Lack of reproducibility and inconsistencies in results depending on testing methods and bacterial growth parameters encompass some of the limitations of the methodology (21–23). Broth dilution is favored, as colistin diffuses poorly in agar, but is cumbersome to perform in a clinical laboratory setting (24, 25). According to the Clinical and Laboratory Standards Institute (CLSI), colistin breakpoint for *Enterobacterales* intermediate susceptibility is defined as an MIC of 2 μg/mL or less, and greater than 2 μg/mL is defined as resistant (26). For the European Committee on Antimicrobial Susceptibility Testing (EUCAST), an MIC of 2 μg/mL or less is defined as susceptible and greater than 2 μg/mL is defined as resistant (27).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is one of the prominent technologies in clinical microbiology laboratories. MALDI-TOF MS provides simple, rapid, and robust sample preparation (28). Two MALDI-TOF MS platforms commonly used in clinical laboratories are the Bruker MALDI Biotyper (MBT) (Bruker Daltonics, Billerica, MA) and bioMérieux Vitek MS (bioMérieux, Inc., Durham, NC) (28). Both protein-based methods utilize an extensive library of mass spectra mainly from ribosomal proteins, highly conserved in bacterial species, as a reference to identify organisms (29). Major drawbacks of these protein-based MALDI-TOF MS platforms are their limited capabilities detecting polymicrobial infections and antimicrobial

resistance (30). However, analyzing glycolipids (lipid-based) by MS adds the ability to detect polymicrobial infections and antimicrobial resistance, especially those affecting the bacterial cell envelope, including colistin, in an easy, rapid, and reproducible manner (13–17, 30).

Previous studies have demonstrated the use of microbial membrane glycolipids for the rapid identification of pathogens, including Gram-negative and Gram-positive bacteria and fungi (13–17, 31, 32). Recently, our group has developed a novel lipid extraction method, fast lipid analysis technique (FLAT), which provides potential utility for clinical microbiology laboratory settings (31). FLAT involves the extraction of lipids directly on a MALDI plate in less than 1 h, significantly reducing labor and handling time compared to those of previous lipid analysis methods. Additionally, FLAT allows for identification of antimicrobial resistance via a MALDI-TOF MS platform. Furthermore, we have shown that modifications to lipid A in the ESKAPE pathogens *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* can be used to determine colistin resistance detection via MALDI- TOF MS analysis. For example, addition of phosphoethanolamine, L-amino-4-arabinose (Ara4N), or galactosamine to terminal phosphates of lipid A reduces membrane electronegativity reducing permeability, thus conferring colistin resistance (13–17). Presence of all these modifications has been observed via MALDI-TOF MS. Finally, mobilized colistin resistance (MCR) genes confer plasmid-mediated resistance to colistin. The mechanism of this resistance is a phosphatidylethanolamine transferase which transfers phosphoethanolamine to lipid A (33). For all Gram-negative ESKAPE pathogens, *mcr* genes and other mechanisms of colistin resistance have been detected and through horizontal gene transfer have rapidly spread to other Gram-negative pathogens. Therefore, a rapid, direct-from-samples analysis is critical for global surveillance of colistin-resistant organisms (16, 34, 35).

Previous studies have confirmed the ability to analyze glycolipids via MS to identify colistin resistance in the other Gram-negative ESKAPE pathogens. This study sought to analyze this using *Enterobacter* species and *Klebsiella aerogenes* after a recent surveillance study by the British Society for Antimicrobial Chemotherapy identified that the annual colistin resistance rates among *E. cloacae* complex isolates were 4.4% to 20%, which was considerably higher that the $<$2% for other Gram-negative *Enterobacteriaceae* organisms (36). Furthermore, this study was the first to confirm the use of FLAT as a rapid, clinically viable assay for the determination of colistin resistance compared to standard broth microdilution AST methods.

RESULTS

***Enterobacter* species clinical isolate overview.** Ninety-eight *Enterobacter* species isolates were collected from 2011 to 2017, of which 74 were *Enterobacter cloacae* complex organisms and 24 were identified as *Klebsiella* (*Enterobacter*) *aerogenes*. For the *Enterobacter cloacae* complex organisms, the following species were identified: *E. cloacae*, *E. asburiae*, *E. hormaechei*, *E. kobei*, *E. ludwigii*, and *E. nimipressuralis* (1). *K. aerogenes* isolates were included in this study since they were initially classified as *Enterobacter aerogenes* and treated as *Enterobacter* infections during the study period. Furthermore, *K. aerogenes* has numerous phenotypic traits shared with *Enterobacter* (37, 38). A schematic breakdown of all clinical isolates, MIC interpretations, and MS results is displayed in Fig. S1.

Determination of MIC levels for *Enterobacter* clinical isolates. MICs ranged from 0 μg/mL to 128 μg/mL with replicate analysis consistent for all isolates. Of the 98 total isolates, 88 were classified intermediate (CLSI) or susceptible (EUCAST) ($\leq$2 μg/mL) and 10 resistant to colistin ($>$2 μg/mL). A breakdown for all isolates was as follows: 10 isolates displayed an MIC of 0.125 μg/mL, 14 of 0.25 μg/mL, 302 of 0.5 μg/mL, 23 of 1 μg/mL, 9 of 2 μg/mL, 1 of 4 μg/mL, 1 of 32 μg/mL, 2 of 64 μg/mL, and 6 of 128 μg/mL.

Observed lipid A modifications in *Enterobacter* species and *Klebsiella aerogenes* by MALDI-TOF MS. As shown in Fig. 1, we identified the base lipid A ion (*m/z* 1,825), which has previously been reported and is indicative of *Enterobacter* species (32). This ion represents a bisphosphorylated hexa-acylated structure (Fig. 2). Additional ions at

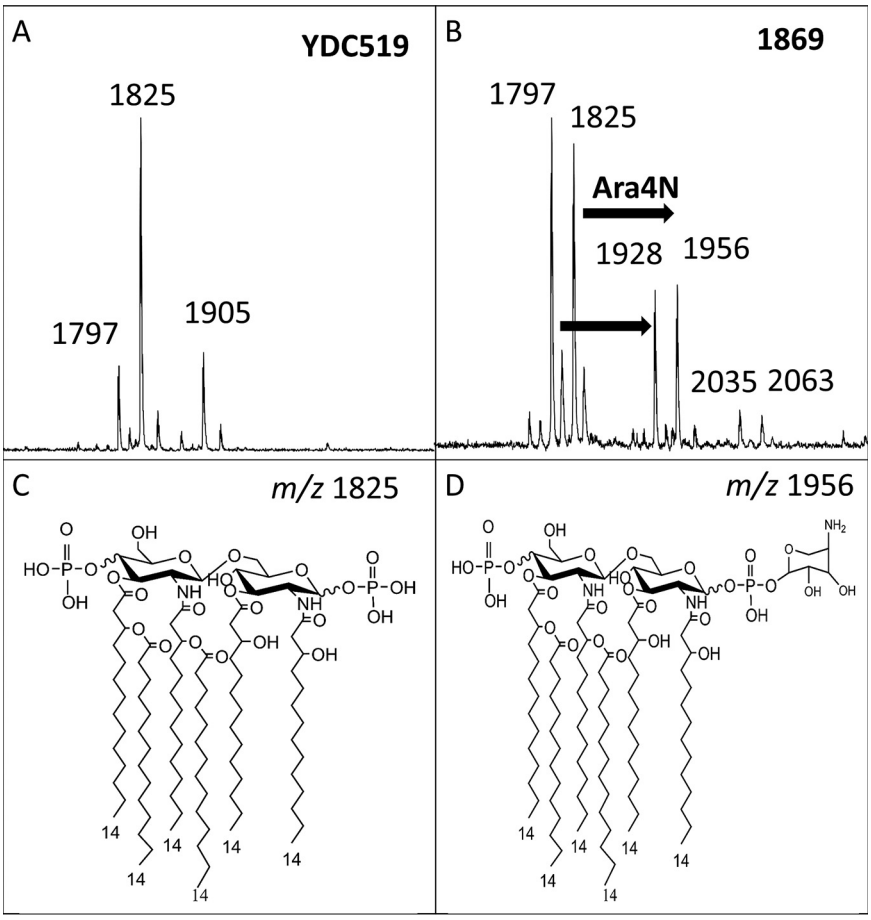


FIG 1 MALDI-TOF MS of *Enterobacter* species and *Klebsiella aerogenes* with difference in colistin susceptibility. Isolates from patients YDC519 and 1869 were grown for 16 h at 37°C on LB agar plates with 2 μg/mL of colistin and without colistin. (A) YDC519 is a colistin-susceptible isolate and has a base lipid A structure consistent with *m/z* 1,825 along with a two-carbon reduction of an acyl chain (*m/z* 1,797) and phosphorylation (*m/z* 1,905). (B) Isolate 1869 is colistin-resistant with *m/z* 1,825 and *m/z* 1,797 as base peaks. Additional ions at *m/z* 1,928 and *m/z* 1,956 indicate an Ara4N addition to base structures. Palmitoylation is shown (*m/z* 2,035, *m/z* 2,063). (C) Lipid A structure for base peak for *Enterobacter*. (D) Lipid A structure commonly found in colistin-resistant isolates.

m/z 1,905 and *m/z* 2,063 represent the addition of a third phosphate ($\Delta m/z$ 80) or palmitate (C_{16}, $\Delta m/z$ 238), respectively (Fig. 2). The minor ions of *m/z* 1,797 and 2,035 represent a two-carbon reduction at the C′-2 acyl-oxo-acyl chain for the respective ions of *m/z* 1,825 and 2,063 (C_2H_4, $\Delta m/z$ 28). The minor ion at *m/z* 1,928 represents a two-carbon reduction at the C′-2 acyl-oxo-acyl chain for the respective ions of *m/z* 1,956 (C_2H_4, $\Delta m/z$ 28). Finally, the ion at *m/z* 1,956 represents the base structure with the addition of an Ara4N moiety ($\Delta m/z$ 131) to one of the terminal phosphate groups (Fig. 1) and is a signature biomarker for colistin resistance. Of the 98 isolates, 51 isolates had the ion at *m/z* 1,956 present.

Correlation of MIC and lipid A modification of *Enterobacter* species. When screening *Enterobacter* species and *Klebsiella aerogenes* isolates by MALDI-TOF MS to identify potential mechanisms of colistin resistance, we observed addition of Ara4N ($\Delta m/z$ 131), a mechanism of colistin resistance that has been reported previously (39). All isolates were screened by MS for presence of Ara4N and compared to current CLSI and EUCAST MIC standard thresholds. For all 10 isolates with a resistant MIC, Ara4N addition was observed by MS analysis. This yielded a sensitivity of 100%, demonstrating that Ara4N is a highly sensitive biomarker for identifying colistin resistance. Of the 88 isolates determined with an intermediate MIC, 41 of the isolates were determined

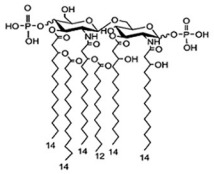
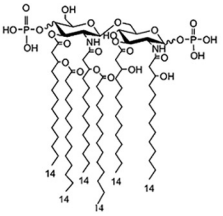
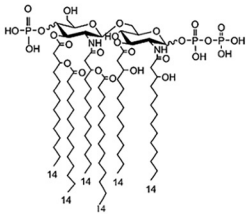
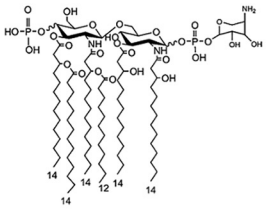
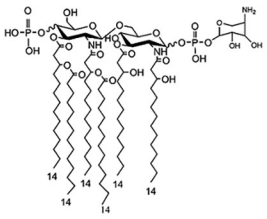
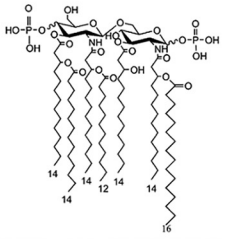
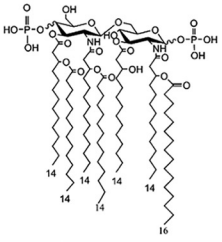
m/z	Proposed Structure	Modification
1797		Reduction of two carbon units of the C'-2 acyl-oxo-acyl chain
1825		Base structure
1905		Phosphorylation of C-1 phosphate group.
1928*		Reduction of two carbon units of the C'-2 acyl-oxo-acyl chain Glycosylation (-Ara4N) of C-1 phosphate group
1956*		Glycosylation (-Ara4N) of C-1 phosphate group
2035		Reduction of two carbon units of the C'-2 acyl-oxo-acyl chain Palmitoylation of the C-1 acyl-oxo-acyl chain
2063		Palmitoylation of the C-1 acyl-oxo-acyl chain

FIG 2 Lipid A structures with respective *m/z* values commonly found in clinical isolates of *Enterobacter* and *Klebsiella aerogenes*. Modifications causing observed mass shifts are described. Asterisks indicate ion associated with colistin resistance.

TABLE 1 Concentrations of colistin to kill 50% of the bacterial population ($kill_{50}$) for all isolates, resistant isolates, and intermediate resistant isolates, comparing isolates with Ara4N and without with 95% confidence interval (CI); furthermore, comparison of median and mean MIC between Ara4N present and absent groups[a]

	Colistin concn μg/mL (95% CI)		
Parameter	**Ara4N present**	**Ara4N absent**	***P* value**
Isolate			
All isolates	6.37 (5.97, 7.71)	0.19 (0.12, 0.28)	<0.001
Resistant (>2 μg/mL)	9.12 (8.21, 12.5)	NA	NA
Intermediate (≤2 μg/mL)	3.55 (2.40, 4.38)	0.19 (0.12, 0.28)	<0.001
MIC			
Median MIC	1.20 (0.50, 1.50)	0.50 (0.12, 1.00)	0.021
Mean MIC	18.8 (7.40, 30.2)	0.69 (0.52, 0.86)	<0.001

[a]For each group, significant differences were seen comparing those with Ara4N to those without ($P < 0.001$).

resistant by MS with Ara4N present, and in 47 isolates the Ara4N was absent and determined intermediate by MS. Based on these results, the specificity for the MS-based colistin assay was 53.4%. Mean and median MICs for the Ara4N present and absent groups were compared. Median MICs for Ara4N present and absent groups were 1.20 (interquartile range [IQR]: 0.50, 1.50) and 0.50 (IQR: 0.12, 1.00), respectively. The mean MICs for Ara4N present and absent were 18.8 μg/mL (95% confidence interval [CI]: 7.40, 30.2) and 0.69 (95% CI: 0.52, 0.86), respectively (Table 1).

Killing assay. Percent survival at different colistin concentrations was plotted to compare isolates with and without Ara4N (Fig. 3). The average concentration of colistin at which there was approximately 50% survival ($kill_{50}$) for all isolates with Ara4N present was 6.37 μg/mL (95% CI: 5.97, 7.71) and 0.19 μg/mL (95% CI: 0.12, 0.28) for those without Ara4N (Table 1). When stratified, intermediate isolates with the modification had an average $kill_{50}$ of 3.55 μg/mL (95% CI: 2.40, 4.38) and 0.19 μg/mL (95% CI: 0.12, 0.28) for those without the Ara4N modification ($P < 0.001$). $Kill_{50}$ for the 10 resistant isolates was 9.12 μg/mL (95% CI: 8.21, 12.49). Plots comparing percent survival at different colistin concentrations for isolates with resistant MICs and Ara4N present, intermediate MICs and Ara4N present, and intermediate MICs and Ara4N absent were significantly different ($P < 0.001$), indicating that the bacterial initial killing kinetics of colistin differ depending on whether Ara4N is present or absent (Fig. 3).

Receiver operator characteristic curves. Receiver operator characteristic curves (ROCs) are commonly used to determine optimal thresholds for diagnostic tests and to measure the overall predictive and diagnostic accuracy of a diagnostic test. In this instance, ROCs were produced to assess and compare the overall predictive and diagnostic ability of the MS-based colistin assay compared to that of $kill_{50}$ and MIC (Fig. 4). The area under the curve (AUC) for $kill_{50}$ and broth microdilution was 0.97 and 0.74,

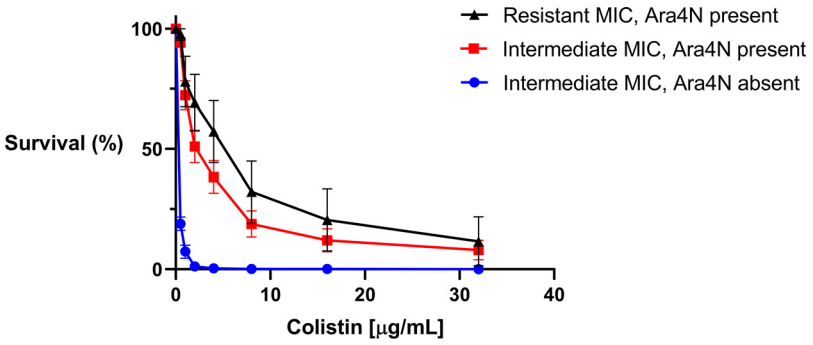


FIG 3 Killing assay comparing intermediate resistant isolates by MIC/Ara4N present (n = 41), intermediate isolates by MIC/Ara4N absent (n = 47), and resistant isolates by MIC/Ara4N (n = 10) present percent survival after 30 min of killing at different colistin concentrations. These curves were statistically different by two-way ANOVA ($P < 0.001$).

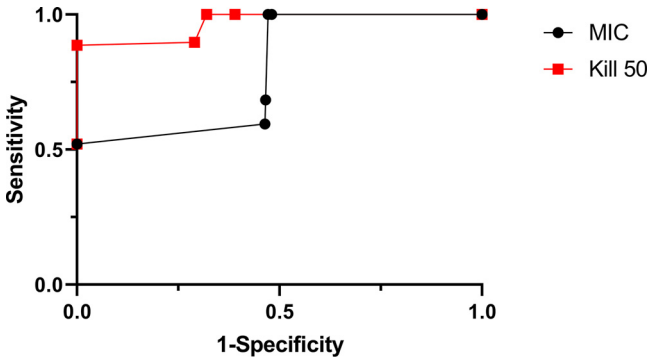


FIG 4 Receiver operator characteristic curves (ROCs) analyzing the diagnostic accuracy of Ara4N compared to MIC (black) and $kill_{50}$ (red). Area under the curve (AUC) for MIC and $kill_{50}$ was 0.74 and 0.97, respectively.

respectively. These results indicate that MS identifies initial killing by colistin more accurately than MIC. Furthermore, an AUC of 0.97 illustrates that the MS-based colistin assay with FLAT extraction is strongly associated with $kill_{50}$ and has diagnostic and predictive value when predicting initial killing of colistin. A perfect predictor or diagnostic has an AUC of 1; with an AUC of 0.97, the MS-based colistin assay is an efficient and accurate alternative to capture initial killing of colistin.

Analysis for MS reproducibility. The FLAT and MS-base colistin assay were determined to be highly reproducible with zero intraday and interday variability observed among the isolates used. For the five isolates with the Ara4N modification, the modification was observed in all three replicates over the course of 5 days. Furthermore, the modifications remained absent for the four isolates previously determined to have lack Aar4N modification. The absence of the modification was observed for all replicates over the course of 5 days, illustrating the high reproducibility of this assay.

DISCUSSION

Enterobacter species and *Klebsiella aerogenes* prominently cause health care-associated infections leading to morbidities, such as sepsis and urinary tract infections (UTI) (2). The increasing incidence of antimicrobial resistance has made combatting *Enterobacter* a top priority by the WHO. To ensure appropriate therapy, it is paramount to develop optimal diagnostic methods. Identification of colistin resistance would further enhance public health outcomes. In this study, using retrospectively collected patient isolates, we observed colistin resistance using MALDI-TOF MS glycolipid analysis, MIC testing, and a colistin killing assay. A strong association between colistin tolerance and an Ara4N addition was observed.

FLAT MS is a highly sensitive method for identification of colistin-resistant *Enterobacter* species and *K. aerogenes*, identifying resistance for 100% of the isolates with resistance MIC interpretations. However, the limitation of this study is the low specificity, 53.4%. The low specificity of this assay can be explained by the several known challenges with polymyxin AST, especially with *Enterobacter* species and *K. aerogenes* (40–44). Broth microdilution is currently the "gold standard" for MIC determination since disc diffusion and Etest have higher false-susceptibility rates due to improper diffusion (43). Despite being the gold standard, broth microdilution is also problematic. One major challenge of broth microdilution is colistin adhering to labware causing inaccurate concentrations of colistin in testing wells. In a study with *Enterobacter cloacae* and *Klebsiella aerogenes*, Landman et al. (44) demonstrate that polymyxin MICs often have low reproducibility and uninterpretable results. This study observed several inaccurate MIC interpretations, including false susceptibilities along with a "skipped well" phenomenon. This phenomenon is characterized by a well

without growth despite growth present at higher concentrations of colistin. A potential explanation for false susceptibilities is the emergence of heteroresistance.

Heteroresistance is defined as having a subpopulation of antibiotic resistance bacteria when the overall population is determined susceptible by *in vitro* methods (45). A public health concern, heteroresistance causes antibiotic treatment failure and negative patient outcomes; thus, it is important to identify (46, 47). Heteroresistance poses a challenge for polymyxin AST, as it leads to misinterpretations and false susceptibilities. Landman et al. demonstrated presence of heteroresistant *Enterobacter* species and a correlation between MIC and proportion of resistant bacteria. Previously, FLAT MS was shown to accurately identify colistin resistance in known heteroresistant populations of *E. coli*, *Klebsiella* species, and *Enterobacter* species (48). False susceptibilities and the ability of FLAT to identify heteroresistance explain the low specificity of this assay compared to that of traditional colistin AST.

Due to the shortcomings of traditional polymyxin AST and the observed discordance in this study, a killing assay was performed to evaluate the pharmacodynamics of colistin when treating potentially resistant bacteria. Polymyxins kill rapidly through interaction and disruption of the bacterial membrane (49). Because of this, the killing assay best reflects pharmacodynamics and response to treatment with colistin, valuable insight for a clinical setting. Additionally, since colistin is nephrotoxic, short-term treatment is ideal (50). Furthermore, the killing assay does not allow adaptation to better capture heteroresistance and provides enumeration so it is not subject to interpretation like MICs (51). Despite providing important clinical information, killing assays are clinically limited due to high labor requirements and lengthy turnaround times; however, the FLAT MS assay is a near-perfect predictor of killing (AUC = 0.97), making it an appropriate clinical substitute. Since $Kill_{50}$ is significantly higher in isolates where the Ara4N is present, this suggests that treatment efficacy would be worse when the marker is detected.

Ideally, a clinical diagnostic platform should be easy to use, rapid, robust, reproducible, and accurate. For these reasons, MALDI-TOF MS is an important resource in clinical microbiology settings (28). The novel extraction method FLAT paired with MS analysis is easy to conduct, robust, highly reproducible, and accurate, and it provides results in under an hour (31) using instrumentation already present in clinical laboratories. Furthermore, FLAT allows for identification of antimicrobial resistance using a MALDI-TOF MS platform. Further development of this lipid-based diagnostic assay for direct-from-sample analysis, using samples such as urine and blood culture bottles, without the need for *ex vivo* culture further highlights the potential clinical utility of our MALDI-TOF MS diagnostic method.

MATERIALS AND METHODS

Bacterial strains. Collection of clinical isolates ($n = 98$) was approved by the University of Pittsburgh Institutional Review Board (PRO12060302). Isolates included in this study were identified by the clinical microbiology laboratory at the University of Pittsburgh Medical Center via MicroScan WalkAway (Beckman Coulter, Brea, CA) and confirmed by MALDI Biotyper research-use-only (RUO) (Bruker Daltonics, Billerica, MA) as *Enterobacter* species upon arrival to the research laboratory (Table S1). Collected isolates were reported as resistant to carbapenems being tested against ertapenem, meropenem, and imipenem. Isolate collection was conducted for 6 years from 2011 to 2017, and these low passage isolates were glycerol stocked and stored at −80°C until MALDI-TOF MS analysis.

Susceptibility testing. MICs were determined by broth dilution method using cationic adjusted Mueller-Hinton broth and conducted following methods published by the CLSI (19). MICs ranged from 0.125 μg/mL to 128 μg/mL. Broth dilutions were replicated 10 times per isolate using separate isolated colonies for each replicate. If there were discordant MICs, broth dilutions were repeated in triplicate. The final MIC was the MIC of the concordant repeated triplicates. Colistin sulfate salt was acquired from Sigma-Aldrich (catalog number C4461; St. Louis, MO). Determination of intermediate and resistant organisms was done using the previously mentioned CLSI breakpoints.

FLAT lipid A extraction and MALDI-TOF MS analysis. Isolates were cultured onto lysogeny broth (LB) plates and grown for 16 h at 37°C. To express resistance, samples were also cultured on LB with 2 μg/mL colistin sulfate plates. Isolates with MICs less than 2 μg/mL had reduced growth. Lipid A analysis was conducted using FLAT per the methods in Sorensen et al. (31). Briefly, a single colony was spotted on a MALDI plate, overlaid with 1 μL of buffer of 0.2 M anhydrous citric acid and 0.1 M trisodium citrate dihydrate, incubated at 110°C for 30 min, and rinsed with endotoxin-free water (31). Each isolate

was spotted in triplicate. Subsequently, 1 μL of 10 mg/mL norharmane matrix suspended in 2:1 chloroform-methanol was spotted onto the extracted lipid sample on a MALDI plate. A Bruker Microflex LRF MALDI-TOF MS in the negative ion and reflectron mode was used to collect mass spectra. The MALDI-TOF MS comprises a 337 nm nitrogen laser. Analyses were conducted at 43% global intensity with approximately 300 laser shots for each spectrum acquisition. Spectra were recorded in triplicate. Electrospray tuning mix (Agilent, Palo Alto, CA) was used for mass calibration. Flex analysis (v3.4) software processed the mass spectra with smoothed and baseline corrections. Signal-to-noise ratios above three were considered optimal for inclusion of signature ions (52).

Killing assay. To determine the $kill_{50}$ for all organisms, individual isolates were grown in 5 mL of lysogenic broth (LB) at 37°C shaking at 180 rpm for 16 h. A subculture was created by adding 250 μL of bacterial culture into 4,750 μL of LB media. Cultures were then grown to 1.5×10^8 CFU/mL (0.5 McFarland standard value) and then diluted to approximately 3×10^5 and 8×10^5 CFU/mL before use. Briefly, 100 μL of this working stock culture was added to 100 μL of LB with colistin at different concentrations and plated in a 96-well round-bottom plate (CELLTREAT Scientific, Pepperell, MA). Concentrations of colistin were 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, 0.5 μg/mL, and 0 μg/mL. Each isolate was plated in triplicate and incubated for 30 min at 37°C to best capture killing at different colistin concentrations, as the bactericidal effect of colistin is extremely rapid. Subsequently, isolates were enumerated for survival by plating on LB and counting cells after 16-h incubation at 37°C. As the negative control, the 0 time point was plated on LB, grown for 16 h at 37°C, and enumerated to measure CFU/mL prior to exposure. $Kill_{50}$ was calculated as follows: cell counts for each isolate at each concentration of colistin were divided by the counts from the 0 time point to calculate percent survival. Percent survival was plotted across each colistin concentration, and the concentration at which there was approximately 50% survival was calculated using nonlinear regression as the $kill_{50}$.

Analysis for MS reproducibility. To evaluate reproducibility of the FLAT procedure for colistin resistance identification in *Enterobacter* species and *Klebsiella aerogenes*, nine isolates, three each with an MIC of 0.5 μg/mL, 2 μg/mL, and 128 μg/mL, were analyzed repeatedly. Of the nine, five showed the presence of Ara4N addition and four lacked this modification during initial analysis. Prior to MS analysis, individual isolates were grown on LB and LB with 2 μg/mL colistin plates (supplemented with 1.5% Bacto-agar). FLAT was conducted for each isolate in triplicate (intraday variability, 27 individual samples) for 5 days (interday variability, 135 individual samples) to evaluate assay reproducibility.

Statistical analyses. Sensitivity and specificity comparing MS (presence or absence of Ara4N) and MIC interpretations (intermediate or resistant) were calculated. Comparison of $kill_{50}$ was conducted for all isolates, intermediate isolates only, and resistant isolates only with and without Ara4N. Percent survival was plotted for each group and compared using a two-way analysis of variance (ANOVA). $Kill_{50}$ was calculated for each above group by pooling data from all isolates in each group and fitting a nonlinear regression. Receiver operator characteristic (ROC) curves were created to compare $kill_{50}$, MIC, and specificity and sensitivity of the MS diagnostic at different colistin concentrations. ROC curves were created using thresholds from each tested concentration of colistin for killing assay and broth microdilution. Isolates with Ara4N present were considered positive for resistance and those with Ara4N absent were considered negative. Statistical analyses and graphs were performed using GraphPad Prism (Version 8). Lipid structures were created using Chem Draw (Version 20).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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