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Evaluation of Sensititre Broth Microdilution Plate for determining the susceptibility of carbapenem-resistant *Klebsiella pneumoniae* to polymyxins☆

Sandra S. Richter[a,*], **A James Karichu**[a], **Joshua Otiso**[a], **Hillary Van Heule**[a], **George Keller**[a], **Eric Cober**[a], **Laura J. Rojas**[b,e], **Andrea M. Hujer**[b,c], **Kristine M. Hujer**[b,d], **Steve Marshall**[b], **Frederico Perez**[b,c,d], **Susan D Rudin**[b,d], **T. Nicholas Domitrovic**[b,d], **Keith S. Kaye**[i], **Robert Salata**[c], **David van Duin**[j], and **Robert A. Bonomo**[b,c,d,e,f,g,h]

[a]Cleveland Clinic, Cleveland, OH

[b]Department of Veteran Affairs Medical Center, Cleveland, OH

[c]Case-VA CARES, Case Western Reserve Univ., Cleveland, OH

[d]Department of Medicine, Case Western Reserve Univ. School of Medicine, Cleveland, OH

[e]Department of Molecular Biology and Microbiology, Case Western Reserve Univ. School of Medicine, Cleveland, OH

[f]Department of Biochemistry, Case Western Reserve Univ. School of Medicine, Cleveland, OH

[g]Department of Pharmacology, Case Western Reserve Univ. School of Medicine, Cleveland, OH

[h]Department of Proteomics and Bioinformatics, Case Western Reserve Univ. School of Medicine, Cleveland, OH

[i]Univ. of Michigan, Ann Arbor, MI

[j]Univ. of North Carolina, Chapel Hill, NC

Abstract

Colistin and polymyxin B MICs were determined for 106 carbapenem-resistant *Klebsiella pneumoniae* (CR-*K*p) isolates using Sensititre Research Use Only GNX2F plates (Thermo Fisher) and compared to CLSI broth macrodilution (BMD) as the reference method. For colistin, EUCAST breakpoints were applied and testing of isolates with very major (VM) errors was repeated in duplicate by both methods to determine a majority result. Essential agreement (MIC ± one dilution) of GNX2F with the reference method was 97.1% for polymyxin B and 92.5% for colistin (7 VM errors, 22.6%). After discrepancy testing, there were 28 colistin resistant isolates by BMD and essential agreement was 94.3% with 4 VM errors (14.3%). Colistin and polymyxin B GNX2F results showed acceptable essential agreement with BMD for MICS without

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*Corresponding author. Tel.: +1-216-444-6519; fax: +1-216-444-7612, richtes@ccf.org (S.S. Richter).

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interpretation. Colistin VM errors with EUCAST breakpoints were due to MIC variability in the 2 to 4 μg/mL range that could be addressed by establishing an intermediate category.

Keywords

Colistin; polymyxin; carbapenem-resistant *Enterobacteriaceae*

1. Introduction

Polymyxins (colistin and polymyxin B) are often prescribed for patients with carbapenem-resistant *Enterobacteriaceae* (CRE), *Acinetobacter baumannii* complex and *Pseudomonas aeruginosa* infections. These agents (cationic antimicrobial peptides) act as positively charged “detergents” interacting with negatively charged lipopolysaccharide (LPS) leading to bacterial membrane leakage and death (Poirel et al., 2017). Bacteria become resistant to polymyxins primarily through modifications in LPS that can be mediated by a variety of genes (Poirel et al., 2017; Baron et al., 2016; Olaitan et al., 2014). A major difference between polymyxins is the manufacturing of colistin (polymyxin E) as a prodrug (colistin methanesulfonate) while polymyxin B is administered in the active form (Nation et al., 2014). Although colistin has been more commonly used, a better pharmacokinetic profile is making polymyxin B the preferred choice for an increasing number of hospital formularies (Kassamali and Danziger, 2015; Vardakas and Falagas, 2017).

With frequent clinical usage and alarm related to the emergence of plasmid-mediated *mcr* resistance (Carattoli et al., 2017; Rolain et al., 2016; Xavier et al., 2016), it is surprising that FDA breakpoints for polymyxins are not established. This prevents manufacturers from providing an FDA approved method to clinical laboratories in the United States. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) proposes colistin breakpoints for *Enterobactericeae* of susceptible, ≤2 μg/mL and resistant, >2 μg/mL. The Clinical and Laboratory Standards Institute (CLSI) has clinical breakpoints for polymyxins with *Acinetobacter* spp. and *P. aeruginosa,* but not *Enterobactericeae* (CLSI, 2017). In 2017, CLSI published an epidemiologic cutoff value (ECV) of ≤2 μg/mL for colistin that applies to five species of *Enterobacteriaceae (Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, Enterobacter aerogenes, Raoultella ornitholytica)* with explicit instructions to not use the ECV as a clinical breakpoint (i.e., do not assign interpretations of “susceptible” to the wild type or “resistant” to the non-wild type population) (CLSI, 2017).

The physical characteristics of polymyxin molecules make determining an accurate MIC challenging. Unacceptably high error rates occur when polymyxins are tested using disk diffusion and gradient diffusion methods (Dafopoulou et al., 2015; Maalej et al., 2011). Isolates tested with surfactant to prevent polymyxin molecules from sticking to the plastic surface in a broth microdilution tray have lower MICs (Hindler and Humphries, 2013; Sader et al., 2012). After much debate, a CLSI-EUCAST working group determined surfactants (e.g., polysorbate-80) should not be included in the reference broth microdilution method when testing colistin (CLSI-EUCAST, 2016). In October of 2016, the American Society for Microbiology cautioned clinical laboratories to limit susceptibility testing for colistin to

broth microdilution methods and to send *Enterobactericeae* isolates with elevated colistin MICs to their public health laboratory for *mcr* screening (ASM, 2016).

Because of the emerging crisis of multidrug resistance (MDR) in Gram negative bacteria, clinicians treat patients infected with CRE, MDR *A. baumannii* complex and MDR *P. aeruginosa* with polymyxins. The outcomes of these “last line treatments” are complicated by many clinical factors. An important group of patients treated with colistin or polymyxin B are enrolled in the Consortium on Resistance Against Car- bapenems in *Klebsiella* and other *Enterobacteriaceae* (CRACKLE) Network, a prospective, multicenter, observational study. We evaluated the accuracy of a commercially available “research use only” (RUO) broth microdilution tray for detecting colistin and polymyxin B resistance in carbapenem-resistant *K. pneumoniae* (CR-*Kp*) isolates.

2. Materials and methods

Colistin and polymyxin B MICs were determined for 106 CR-*K*p isolates using the Sensititre™ Research Use Only GNX2F plate (Thermo Fisher) and compared to previously determined CLSI broth macrodilution results as the reference method. Surfactant (e.g., polysorbate 80) was not used in either method. The *K. pneumoniae* isolates were recovered from clinical specimens of unique patients cultured during December 2011 to October 2014 and submitted to the CRACKLE surveillance program (Rojas et al., 2017; van Duin et al., 2014). The mechanism of carba- penem resistance was $bla_{\text{KPC-2}}$ for 46% and $bla_{\text{KPC-3}}$ for 50% of the CRACKLE isolates; isolates with a colistin broth macrodilution MIC in the resistant range (EUCAST breakpoints applied) were screened and did not carry *mcr-1* or *mcr-2* (Rojas et al., 2017).

Testing on the GNX2F plates was performed at the Cleveland Clinic Microbiology Laboratory. Sensititre panels are plastic (virgin polystyrene) micro-titer plates containing dried antimicrobial agents. After two subcultures from frozen stock cultures stored at −70°C, Sensititre GNX2F trays were inoculated according to the manufacturer’s recommendations using cation-adjusted Mueller-Hinton broth. Endpoints were determined after incubation in ambient air for 24 hours. Quality control was performed with *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

The broth macrodilution reference method was performed in duplicate at the CRACKLE reference laboratory in Cleveland according to CLSI M7 guidelines using polymyxin B (Sigma-Aldrich) and colistin (sulfate salt; Sigma Aldrich) with *E. coli* ATCC 25922 for quality control (CLSI, 2015). Fisherbrand disposable 16×100mm culture tubes made of boro-silicate glass (Fisher Scientific) were used. Testing on isolates with broth macrodilution results differing by greater than one dilution was repeated to determine a majority result.

For colistin, EUCAST breakpoints were applied and testing of isolates with very major (VM) errors (GNX2F MIC susceptible, reference method resistant) were repeated in duplicate from the same subculture by both methods to determine a majority result. The VM error rates were calculated using the number of resistant isolates as the denominator.

3. Results

Comparison of the colistin MICs determined with the Sensititre GNX2F plate to reference broth macrodilution results are shown in Table 1. Initial results indicated 31 isolates were resistant to colistin by the reference method. For most isolates (92%), the reference colistin MICs were ≤0.5 μg/mL or ≥8 μg/mL. The initial essential agreement of GNX2F colistin results with the reference method was 91.5% (97/106) with 7 VM errors (22.6%) represented by reference MICs ≥4 μg/mL and GNX2F results of ≤0.25 μg/mL (3 isolates) or 2 μg/mL (4 isolates). After repeat testing by both methods of isolates with VM errors, the number of isolates resistant to colistin by the reference method was reduced to 28 isolates with colistin essential agreement of 94.3% (100/106) and 14.3% VM errors. The 4 isolates that demonstrated VM errors had GNX2F colistin MICs of 2 μg/mL and the reference MICs were only one dilution higher for 3 of those 4 isolates.

The polymyxin B MICs determined using the Sensititre GNX2F plate are compared to the reference broth macrodilution results in Table 2. Two isolates with Sensititre GNX2F MICs of 1 and 2 μg/mL, respectively, but variable BMD results (2,>8,1, 0.25 μg/mL; 8,1, 2, >8 μg/mL) were not included in the comparison. Essential agreement (MIC ± one dilution) of GNX2F polymyxin B results with the reference method was 97.1% (101/104). Two isolates with reference polymyxin B MICs of >8 μg/mL had GNX2F MIC of 0.25 and 1 μg/mL, respectively. One isolate with a reference polymyxin B MIC of 1 μg/mL had a GNX2F MIC of <0.25 μg/mL. Discrepancy testing after GNX2F testing was not performed for polymyxin B, so it is unknown if repeat testing of both methods using the same subculture would have improved the agreement.

4. Discussion

This study provides important performance data for a commercially available RUO broth microdilution method of testing colistin using the largest collection of CR-*K*p and colistin-resistant isolates published to date. These isolates represent bla_{KPC}-positive *K. pneumoniae* which are the most common species of CRE encountered globally. The high mortality associated with invasive CRE infections, especially bloodstream infections and pneumonias, (Hauck et al., 2016; Patel et al., 2008) underscores the importance of having reliable susceptibility testing methods readily available in the clinical laboratory. Although VM error rates for colistin were elevated for GNX2F when EUCAST breakpoints were applied, there were fewer errors than previously reported with Etest (VM error rate of 35%, major error rate of 0.4%) (Rojas et al., 2017).

A study performed on 41 carbapenem non-susceptible *K. pneumoniae* from Greek hospitals reported 41.5% VM errors when testing colistin with Etest compared to CLSI broth microdilution and no errors for the Vitek2 AST-EXN8 card available outside of the US (Dafopoulou et al., 2015). A smaller US study reported that VM errors were not observed for colistin when testing 20 *K. pneumoniae* isolates on Sensititre GNXF trays (Hindler and Humphries, 2013).

A recent analysis performed on 76 CRE clinical isolates from patients in Singapore (including only 6 KPC positive *K. pneumoniae*) compared Sensititre GNX3F to broth microdilution with essential agreements of 89.5% and 96.1% for colistin and polymyxin B, respectively and one VM error which occurred when testing an *E. cloacae* complex isolate (Chew et al., 2017). The study included 21 *Enterobactericeae* (18 *Escherichia coli,* 2 *K. pneumoniae,* 1 *Enterobacter aerogenes)* with *mcr-1* and found 3 carbapenemase positive *E. coli* isolates with colistin broth microdilution MICs of 2 μg/mL which would be considered susceptible by the EUCAST breakpoint of ≤2 μg/mL and wild type by the CLSI ECV (Chew et al., 2017).

More pharmacokinetic/pharmacodynamic and clinical outcome data are needed to determine optimum polymyxin breakpoints for single agent therapy (Bergen et al., 2012; Garonzik et al., 2011; Tran et al., 2016). Population analysis profiling has demonstrated that clinical isolates of *K. pneumoniae* with a susceptible colistin MIC may harbor a subpopulation of resistant isolates (Halaby et al., 2016; Meletis et al., 2011) and combination therapy may prevent the emergence of resistance (Poudyal et al., 2008). Heteroresistance may explain the variable broth microdilution polymyxin B MIC results observed for the 2 isolates excluded from the method comparison.

The failure of CLSI to adopt the EUCAST colistin breakpoints for *Enterobactericeae* suggests they need to be changed to better predict clinical response. The colistin VM errors caused by MIC variability in the 2 to 4 μg/mL range could be addressed by establishing an intermediate breakpoint category. The initial VM error rates for colistin based on a comparison to historical broth macrodilution results would have been 7.1% (2/28) if an intermediate category of 2–4 μg/mL were applied and the EUCAST susceptible breakpoint lowered to ≤1 μg/mL. There would be no VM errors for the final results where both methods were performed from the same subculture plate if these hypothetical breakpoints (intermediate, 2–4 μg/mL; susceptible, ≤1 μg/mL) were applied. The minor error rates with these breakpoints would be acceptable at 6.6% and 5.7% for initial and final results, respectively.

A limitation of the current study is the comparison of GNX2F results to broth macrodilution MICs determined at an earlier point in time at a different laboratory. Discrepancy testing was performed for colistin results to address this issue. Fresh subcultures of isolates with VM errors were transported to the reference laboratory for repeat broth macrodilution testing from the same plate used for repeat GNX2F testing. Possible reasons for changes in reference broth macrodilution results when discrepancy testing was performed by both methods from the same subculture include loss of resistance due to freeze- thaw effect, a banking error, or heterogeneity of resistance (Halaby et al., 2016).

In conclusion, the colistin and polymyxin B MICs of CR-*Kp* from the GNX2F tray had acceptable essential agreement compared to the CLSI broth macrodilution reference method. This RUO plate reliably identifies most isolates with low or high polymyxin MICs. After the appropriate laboratory validation, the GNX2F can provide helpful information to clinicians treating CRE infections and identify isolates to screen for *mcr*-mediated resistance. Clinical breakpoints that would allow microbiology laboratories in the US to report an interpretation

are needed. Performance of the GNX2F would not be acceptable for laboratories applying EUCAST colistin breakpoints for *Enterobacteriaceae.* An intermediate category to address variability of MICs in the 2 to 4 μg/mL range would help manufacturers develop devices for testing colistin that can attain regulatory approval. In the meantime, laboratories reporting GNX2F MICs without an interpretation may consider adding a comment indicating the variability of colistin MICs in the 2–4 μg/mL range. Sending CRE out to a reference laboratory for determination of polymyxin MICs delays reporting of results needed for patient care and epidemiologic surveillance.

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Table 1

Comparison of colistin MICs determined by Sensititre and the CLSI broth macrodilution reference method

Sensititre MIC (μg/mL)		No. isolates with initial/final colistin reference MIC (μg/mL)[a]					
	No. isolates	≥0.5	1	2	4	8	>8
<0.25	74	67/69	2	2[b]/3[b]	1[b,c]/0	2[c,d]/0	-
0.5	4	4	-	-	-	-	-
1	-	-	-	-	-	-	-
2	4	-	-	-	2[c]/3[c]	1[b,c]/1[b,c]	1[b,c]/0
4	2	-	-	-	-	2[b]	-
>4	22	-	-	-	-	1	21
Total	106	71/73	2	2/3	3	6/4	22/21

[a] Determined by CLSI broth macrodilution method.

[b] Minor errors if a susceptible breakpoint of ≤1 μg/mL, an intermediate breakpoint of 2–4 μg/mL, and a resistant breakpoint of >8 μg/mL were applied.

[c] Very major errors if EUCAST breakpoints (≤2 μg/mL, susceptible; ≥4 μg/mL, resistant) were applied.

[d] Very major errors if a susceptible breakpoint of ≤1 μg/mL, an intermediate breakpoint of 2–4 μg/mL, and a resistant breakpoint of ≥8 μg/mL were applied.

Table 2

Comparison of polymyxin B MICs determined by Sensititre and the CLSI broth macrodilution reference method

Sensititre MIC (μg/mL)		No. isolates with polymyxin B reference MIC (μg/mL)[a]					
	No. isolates	<0.5	1	2	4	8	>8
<0.25	65	63	1	-	-	-	1
0.5	13	13	-	-	-	-	-
1	3	1	-	1	-	-	1
2	-	-	-	-	-	-	
4	2	-	-	-	-	2	-
>4	21	-	-	-	-	1	20
Total	104	77	1	1	0	3	22

[a]Determined by CLSI broth macrodilution (BMD) method. Two isolates with Sensititre MICs of 1 and 2 μg/mL, respectively, but variable BMD results were not included in the table. The BMD MICs of those isolates were 2, >8,1, 0.25 μg/mL and 8,1, 2, >8 μg/mL, respectively.