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Colistin susceptibility test evaluation of multiple-resistance-level *Pseudomonas aeruginosa* isolates generated in a morbidostat device

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Objectives: Colistin is a last-resort antibiotic against the critical-status pathogen *Pseudomonas aeruginosa*. There is still uncertainty regarding how to accurately measure colistin susceptibility in *P. aeruginosa*. Evaluation of antimicrobial susceptibility testing (AST) methods is largely hampered by the lack of resistant isolates and those around the susceptibility breakpoint. The aim of this study was to generate such strains in a morbidostat device for use in AST method evaluation.

Methods: A morbidostat device was used to cultivate susceptible clinical strains into isolates with a wide range of colistin MICs. Subsequently, five commercial AST methods were compared against the gold standard broth microdilution (BMD) method: MICRONAUT-S, SensiTest, Sensititre, Rapid Polymyxin Pseudomonas and Etest.

Results: A total of 131 *P. aeruginosa* isolates were used for colistin susceptibility test evaluation (100 colistin susceptible and 31 colistin resistant). The 31 colistin-resistant isolates evolved resistance in the morbidostat to different MIC ranges (4–512 mg/L, 100% resistance generation efficacy). The categorical agreement (CA) rates for MICRONAUT-S, SensiTest and Rapid Polymyxin Pseudomonas were 94.7%, 93.9% and 92.4%, respectively. The Sensititre achieved the highest CA score (96.9%), whereas the Etests had the lowest CA score (84%). The very major discrepancy (VMD) rates for all tests were between 3.2% and 67.7%.

Conclusions: The morbidostat device can efficiently provide laboratories with colistin-resistant strains for test evaluation. Although CA rates were high for commercial AST methods except for Etests, none met the $\leq$1.5% CLSI limit for VMD rates. Performance was generally inferior when using isolates with low-level resistance.

Introduction

The WHO has listed *Pseudomonas aeruginosa* as one of the critical pathogens in urgent need of new antibiotics.[1] Infections with *P. aeruginosa* are associated with high mortality and morbidity, particularly in immunocompromised patients.[2] XDR strains of this bacterium are often susceptible to only colistin, which is now established as a last-resort drug.[3] Colistin is a broad-spectrum antibiotic that is part of the polymyxin family. It is effective against Gram-negative organisms such as *P. aeruginosa*, but has no activity against Gram-positive bacteria. The rising emergence of resistance to colistin, and concerns of toxicity in therapy, makes it crucial to use a fast and reliable antimicrobial susceptibility testing (AST) method to categorize isolates in case treatment becomes necessary.[4,5]

EUCAST advised against the use of gradient and disc diffusion tests for colistin.[6] In 2016, the CLSI and EUCAST recommended the ISO-20776 standard broth microdilution (BMD) method for MIC determination of colistin, with freshly prepared or frozen antibiotic.[7] However, BMD methods are impractical for diagnostic laboratories owing to the considerable in-house preparation required, leading to high workload. Several commercial products are now on the market that exploit the same principle as the BMD, but in a more convenient and user-friendly format.

Colistin susceptibility testing has varied in previous studies with Gram-negative bacteria, likely owing to a number of difficulties encountered in susceptibility testing with colistin: namely the use of different susceptibility breakpoints, the cationic nature of colistin, its large molecular size and heteroresistance.[8–10]

Another important reason for the observed heterogeneity could be the MIC range of the study strains. Selecting isolates at extreme ends of the MIC scale will reduce the rate of errors, whereas a selection of isolates straddling the breakpoint will result in a higher

proportion of errors. Based on the current literature, it is likely that the rate of significant errors is being underreported, owing to the small number of resistant isolates and those around the susceptibility breakpoint being included in comparing AST methods. One reason for this is the fact that colistin-resistant isolates of *P. aeruginosa* have low prevalence in the clinic or remain undetected owing to the pitfalls of susceptibility testing. Thus, investigation of colistin AST methods is commonly outside the scope of a single laboratory. Such studies are usually only feasible for national reference laboratories that hold comprehensive biobanks of strains that have been collected over many years from various laboratories. In order to overcome these limitations and to enable evaluation of AST methods before low-frequency resistance becomes far more prevalent and a highly relevant clinical issue, we used a device called a morbidostat to acquire *P. aeruginosa* strains with distinct levels of colistin resistance. The morbidostat is a continuous-culture automated device that can grow bacteria under constant selection pressure and forces them to develop resistance in cases where resistance is mediated by chromosomal alterations. Our group has previously reported that colistin resistance-conferring mutations acquired in the morbidostat are very similar to those observed in colistin-resistant clinical isolates of *P. aeruginosa*.[11] For these reasons, the morbidostat can be used to transform colistin-susceptible clinical isolates into resistant ones, thereby resembling a 'natural' acquisition of resistance. With this methodology, a sufficiently large strain collection can be generated, comprising isolates with different levels of resistance.

The objective of this study was to generate a comprehensive collection of colistin-resistant *P. aeruginosa* strains and to compare and evaluate three commercial BMD products, the gradient Etest and the colorimetric reaction-based Rapid Polymyxin Pseudomonas (Rapid PP) test with the reference BMD method.

Materials and methods

Genomic and phenotypic characterization of the colistin-susceptible clinical strains

A starting total of 87 colistin-susceptible strains from a collection of *P. aeruginosa* strains isolated from patients with bloodstream infection was chosen for the study. Species identification was performed using MALDI-TOF MS and the VITEK 2 system (bioMérieux, Marcy l'Étoile, France). Antibiotic susceptibility for 15 antibiotics was assessed by Etest and strains were categorized according to their resistance profile.[12] In order to determine genetic relatedness, all strains were whole-genome sequenced on a HiSeq 2500 platform (Illumina, San Diego, USA) using a 2×125 bp approach. SPAdes (version 3.7.0)[13] was applied as a *de novo* assembly tool and a core genome was constructed by Spine (version 0.1.2).[14] SNPs were subsequently called using SAMtools (version 0.1.19)[15] and a pairwise SNP distance matrix was constructed by MEGA (version 7.0.26).[16]

Generation of colistin-resistant isolates in the morbidostat

The protocols for building and using a morbidostat are described elsewhere in great detail.[11,17] Eighteen of the above-mentioned strains were continuously cultivated for up to 58 days in the morbidostat with colistin, starting with 4 mg/L and with a final concentration of 500 mg/L. Figure 1 demonstrates a breakdown of the isolates in our study. Samples of the cultures were taken every 2–3 days. To ensure replicability of AST methods, each isolate was grown on a range of blood agar plates containing 2 mg/L, 8 mg/L, 16 mg/L, 32 mg/L or 64 mg/L of colistin. This was to ensure a truly resistant population, devoid of persister cells or dormant bacteria. One isolate from the colistin-containing blood agar plate was taken and grown overnight on plain blood agar plates at 37°C. These colonies were frozen via the Microbank system (Pro-Lab Diagnostics Inc, Texas, USA). All AST methods were performed with one isolate taken from these frozen stocks and grown overnight on plain blood agar plates. The ability of the morbidostat to pressure susceptible isolates into developing resistance is termed the generation efficacy rate and is calculated by measuring the number of strains that become resistant out of the number of colistin-susceptible strains used. The overall approach of generating resistant isolates was approved by our local ethics review committee (489/2017BO2).

BMD

All isolates were tested for colistin susceptibility using BMD, performed according to ISO standard 20776-1.[7] Briefly, colistin sulphate (Fagron GmbH, Rotterdam, The Netherlands) stock was prepared by dissolving in sterile distilled water to a concentration of 10 000 mg/L and further diluted to the required concentrations with CAMHB (BD, Franklin Lakes, USA). BMDs were carried out in 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany). *P. aeruginosa* ATCC 27853 was used as the colistin-susceptible quality control strain and the MIC was determined to be 2 mg/L by the reference BMD method. BMD was performed on each isolate in triplicate and the median was applied as the final MIC value.

The inter- and intra-assay repeatability and precision of the BMD was also measured. A representative sample of eight strains were selected, including the quality control strain ATCC 27853: four with an MIC value between 1–2 mg/L and four with MIC values of 4–16 mg/L. A BMD was performed on three separate days by two independent observers, with six replicates per strain.

AST comparison and data interpretation

MICRONAUT-S (MERLIN Diagnostika GmbH, Bornheim, Germany), SensiTest Colistin (Liofilchem, Roseto degli Abruzzi, Italy), Sensititre (colistin only panel; Thermo Fisher Scientific) and Rapid PP (ELITech Group, Paris, France) tests were carried out according to the manufacturers' instructions. Etests were also carried out according to the manufacturer's instructions; however, they were read at two timepoints: 24 h and 48 h. The hands-on time was calculated as the average time taken to test one strain, including preparation time.

Colistin breakpoints for *P. aeruginosa* were applied, as proposed by EUCAST (susceptible $\leq$2 mg/L, resistant >2 mg/L).[18] The ranges of colistin MICs available within each testing method are as listed: BMD 0.25–512 mg/L; Etest 0.016–256 mg/L; MICRONAUT-S 0.25–64 mg/L; SensiTest 0.25–16 mg/L; Sensititre: 0.25–128 mg/L; and Rapid PP 2–8 mg/L.

The resulting MICs from each test were organized into four categories, calculated as a percentage relative to the reference BMD method.[19] Categorical agreement (CA) is defined as the qualitative interpretation of MIC from the testing method agreeing with the reference BMD method; the bacteria are categorized as either susceptible or resistant. Essential agreement (EA) is achieved when the MIC result from the testing method is within $\pm$1 dilution step of the reference BMD method. For all tests, the calculation for the EA rate was adjusted owing to the range of MIC values that each testing method provides, by changing the total number of isolates in the denominator. A major discrepancy (MD) occurred when the isolate was categorized as resistant and the reference BMD method indicated it was susceptible. This was calculated with the number of susceptible isolates as the denominator. The very major discrepancy (VMD) category is defined as the MIC result from the testing method being categorized as susceptible to colistin when the reference BMD method indicates that it is resistant, calculated with the number of resistant isolates used as

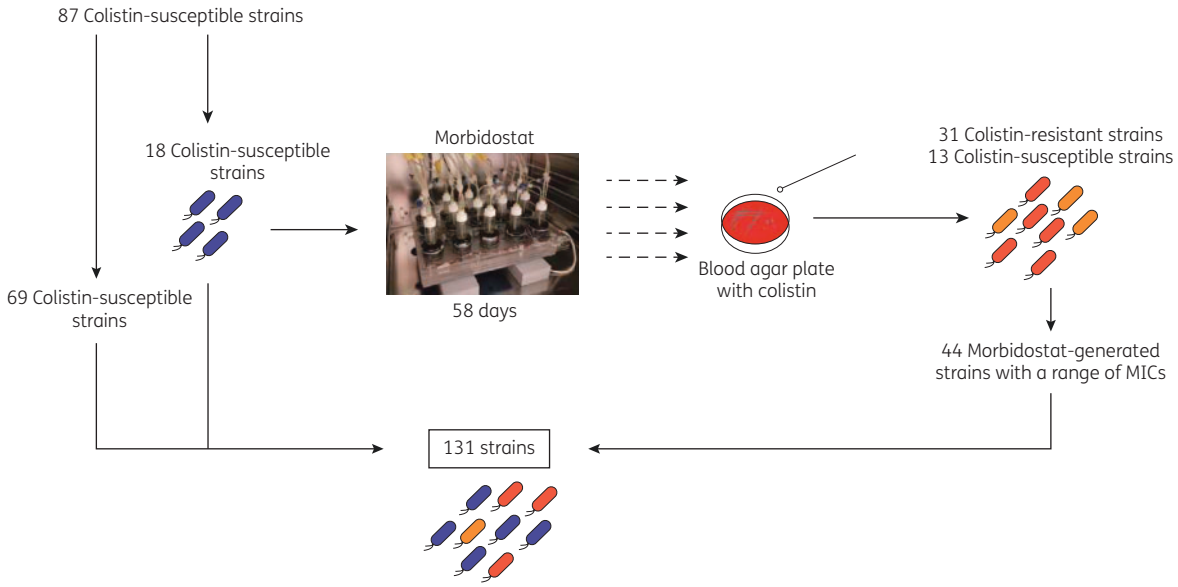

Figure 1. Breakdown of the 131 *Pseudomonas aeruginosa* strains selected for colistin susceptibility test evaluation, including cultivation in the morbidostat device for up to 58 days. Susceptibility to colistin is defined as MIC $\leq$ 2 mg/L and resistance to colistin is MIC > 2 mg/L. The rod shapes represent *P. aeruginosa* strains. Blue strains: clinical isolates susceptible to colistin. Orange strains: strains derived from the morbidostat with an increased MIC value but still susceptible to colistin. Red strains: strains derived from the morbidostat and resistant to colistin. The broken arrows represent strains that have been taken from the morbidostat every 2–3 days and plated on blood agar plates supplemented with colistin. Cultivation in the morbidostat took up to 58 days.

the denominator. The Rapid PP was not included in EA categories owing to the limited range of MICs that can be measured.

All colistin-resistant isolates were separated into three resistance levels: low (4–8 mg/L), medium (16–64 mg/L) and high (128–512 mg/L).

Statistical analysis

All statistical analyses were completed using GraphPad Prism version 6 (GraphPad Software, San Diego, USA) and Stata version 12.1 (Stat Corp., College Station, USA). A Spearman's rank correlation was applied to measure the association between the MIC results of each AST method and the BMD. We used a two-way random-effects model to compute the intraclass correlation coefficient (ICC) in order to compare different observers as well as the results of the same observer but from different days. A *P* value of $\leq$0.05 was considered significant. The coefficient of variation (CoV) was calculated by dividing the SD from a replicate series by the mean of the same series and multiplying the quotient by 100.

Results

Genomic structure and antibiotic susceptibility of the study isolates

Eighty-seven clinical colistin-susceptible strains were initially selected for the study and were sequenced. Each strain was genetically distinct from the others to ensure a representative sample was tested (Table S1, available as Supplementary data at *JAC* Online). The minimum pairwise distance between all isolates was 183 SNPs and the maximum was 72 478 SNPs. The median genetic distance was 23 162 SNPs. There was no presence of *mcr* genes in any of the strains. Etests were completed with 15 antibiotics to measure the susceptibility of each strain (Table S2). The isolates were also categorized according to their resistance profile:[12] 53% non-MDR, 41% MDR, 6% XDR and 0% pandrug resistant (PDR).

Resistance generation efficacy of the morbidostat

From the 87 colistin-susceptible strains, we selected 18 for cultivation in the morbidostat, with the aim of generating resistant isolates with a range of colistin MICs (Figure 1). These 18 strains were genetically diverse (Table S1) and susceptible to colistin. At various points over 58 days, all 18 strains became resistant to colistin, resulting in a 100% resistance generation efficacy rate by the morbidostat.

We selected 31 colistin-resistant isolates from the morbidostat. In order to test the ability of AST methods to accurately distinguish between susceptibility and resistance, we additionally selected 13 isolates from the morbidostat that had increased in their MIC value but were still susceptible. These isolates showed some changes towards resistance, but had not achieved full resistance to colistin (n = 1 with MIC = 1 mg/L, n = 12 with MIC = 2 mg/L). In total, we used 131 *P. aeruginosa* isolates for AST testing (87 clinical strains and 44 morbidostat-generated strains).

Colistin susceptibility of the study isolates

The range of colistin MICs, according to the BMD method, for all 131 isolates is presented in Figure 2 and Table S3(a). Colistin MICs were determined to be between 0.25 and 512 mg/L (MIC 0.25 mg/L, n = 2; MIC 0.5 mg/L, n = 8; MIC 1 mg/L, n = 36; MIC 2 mg/L, n = 54; MIC 4 mg/L, n = 3; MIC 8 mg/L, n = 7; MIC 16 mg/L, n = 6; MIC 32 mg/L, n = 4; MIC 64 mg/L, n = 8; MIC 128 mg/L, n = 1; and MIC 512 mg/L, n = 2). One hundred isolates (76%) were within the

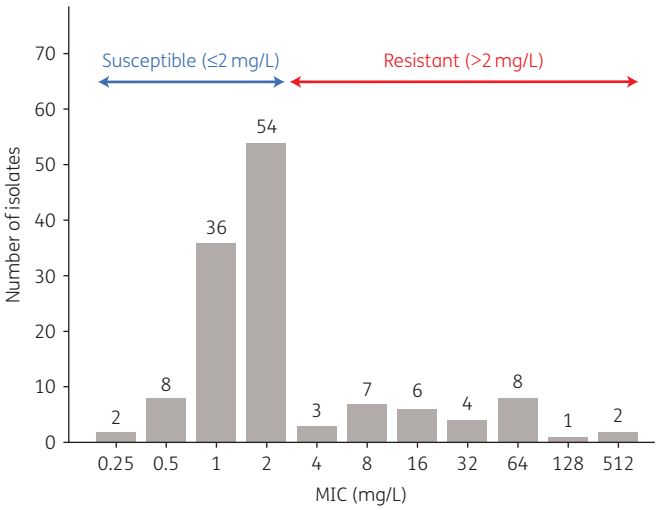

Figure 2. Distribution of colistin MIC determination and susceptibility status with reference BMD method. The values above the columns represent the number of isolates. The double-headed arrows above the columns indicate whether an isolate was susceptible or resistant to colistin.

susceptible range and 31 strains (24%) were categorized as resistant. The MIC_{50} for the 87 colistin-susceptible strains was calculated as 1 mg/L and as 8 mg/L for the morbidostat-derived strains. The MIC_{90}s for colistin-susceptible and morbidostat-derived strains were 2 mg/L and 64 mg/L, respectively. The MIC_{50} for all 131 strains was 2 mg/L and the MIC_{90} was 32 mg/L. Further details on the origin and morphology of the 31 colistin-resistant strains are provided in Table S3(b).

Inter- and intra-assay repeatability and precision for the in-house BMD method

We have tested the validity of our in-house BMD, which has been used as the gold standard in our study. Raw data from the measure of validity, using a representative set of *P. aeruginosa* strains, including both colistin-susceptible and -resistant strains, is shown in Table S3(c). Two observers independently measured the MICs of eight strains on three different days, performing six replicates per strain. When comparing the results of both observers, an ICC of 0.99 ($P < 0.001$) was observed, indicating a high level of concordance between the observers. Repeatability was measured by comparing the MIC results of each observer from three different days. An ICC of 0.89 ($P < 0.001$) was computed for the first observer and an ICC of 0.93 ($P < 0.001$) for the second observer. These values demonstrate a high repeatability of the test results. The mean CoV for both observers was 11.05% (median CoV = 0%), illustrating a high degree of repeatability and precision for each experiment.

Performance of commercial AST methods

The performance of each method relative to the reference BMD is presented in Table 1 and the MIC values resulting from each AST method in Table S3(a). The CLSI has established a set of minimum requirements that each testing method must meet in order to be recommended for use (CA ≥90%, EA ≥90%, MD ≤3.0% and VMD ≤1.5%).[19] These requirements have been applied to our results.

The correlations between Etest at both 24 and 48 h timepoints and the BMD are significantly lower than between other AST methods and BMD (Spearman's $r = 0.478$ and 0.470, respectively). Etests also failed to achieve the ≥90% standard required for CA, with 84.0% at 24 h and 84.7% at 48 h. The EA rate was 70.5%–72.0% for both 24 and 48 h timepoints. The VMD rate was 67.7% and 61.3%, at 24 and 48 h respectively, indicating that Etests are unable to recognize resistant isolates. There were no MD errors for the Etest at 24 h, and at 48 h the MD rate was 1.0%. The Etest had one of the lowest hands-on times, with 25 min taken on average to test five isolates (Table S4).

Of the commercial BMD methods, the Sensititre fared the best, meeting the CLSI requirements for three out of four categories (CA, EA and MD). The results of this method correlated well with the BMD (Spearman's $r = 0.728$). The MICRONAUT-S achieved 94.7% CA. However, it failed to meet the CLSI requirements in other areas, particularly EA (86.7%) and VMD (6.5%) (Spearman's $r = 0.731$). The SensiTest had an EA rate of 88%, with 102/116 isolates within a 2-fold dilution of the BMD results (Spearman's $r = 0.692$). The VMD rate was 6.5%.

The Rapid PP achieved a CA rate of 92.4%, categorizing 121/131 isolates correctly. Although it recorded the highest number of false-positive results (MD of 9.0%), it only showed one VMD. The hands-on time for this test was 25 min to test five isolates, including preparation time (Table S4). Most notably, test results were available to read after 4 h, compared with 18–24 h for all other tests.

Evaluation of AST methods with different levels of resistance

To further understand the dynamics between results of the testing methods, we divided the MICs of resistant isolates into three resistance levels: low (4–8 mg/L), medium (16–64 mg/L) and high (128–512 mg/L), as presented in Table 2. The Etest (24 h) only categorized 2/10 isolates correctly in the low-resistance division. The CA rate for the other tests in the low-resistance division fell to 80% (also below the CLSI minimum), except for Rapid PP, which achieved 90%. The EA rate dropped for the SensiTest in the low-resistance division compared with the total isolates, achieving 50%, compared with 88.0% for all isolates combined. This pattern was replicated for the two other commercial BMD products: the MICRONAUT-S and Sensititre achieved EA rates of 86.7% and 90.7% for all isolates, which decreased to 50% and 70%, respectively, in the low-resistance division. The VMD rate increased significantly when isolates with low resistance were tested: the MICRONAUT-S, SensiTest and Sensititre yielded a 20% VMD rate. The Rapid PP achieved the lowest VMD rate, at 10% (1/10). There were no VMD errors, and a 100% CA rate, for commercial BMD methods using medium-resistance isolates.

The isolates were also divided into susceptible and resistant isolates (Table S5). In the susceptible division, the Etest achieved a CA rate of 100% concordance with the BMD at 24 h. Etests at 48 h had a CA rate of 99%. With the resistant isolates, however, the CA rate decreased to 38.7%, further supporting the view that Etests are unable to distinguish between resistant and susceptible isolates. The commercial BMD tests achieved similar results with susceptible and resistant isolates, with a 91%–98% CA rate, with the

Table 1. Performance of each AST method compared with the reference BMD method

AST method	Percentage of tests (*n*/*N*)				Spearman's *r*
	CA	EA[a]	MD[b]	VMD[c]	
Etest (24 h)	84.0 (110/131)	70.5 (91/129)	0.0 (0/100)	67.7 (21/31)	0.478
Etest (48 h)	84.7 (111/131)	72.0 (93/129)	1.0 (1/100)	61.3 (19/31)	0.470
MICRONAUT-S	94.7 (124/131)	86.7 (111/128)	5.0 (5/100)	6.5 (2/31)	0.731
Sensititre	96.9 (127/131)	90.7 (117/129)	2.0 (2/100)	6.5 (2/31)	0.728
SensiTest	93.9 (123/131)	88.0 (102/116)	6.0 (6/100)	6.5 (2/31)	0.692
Rapid PP[d]	92.4 (121/131)	NA	9.0 (9/100)	3.2 (1/31)	NA

NA, not applicable.
[a]EA rate for each testing method was adjusted to account for the MIC range that is available on the testing panel. For example, the MICRONAUT-S test is able to evaluate MICs between 0.025 and 64 mg/L so only isolates in this range are included in the evaluation for this category.
[b]The MD is calculated as a percentage relative to the BMD testing results, with the number of susceptible isolates as the denominator.
[c]The VMD is calculated as a percentage relative to the BMD testing results, with the number of resistant isolates as the denominator.
[d]The Rapid PP was not included in evaluation of EA and Spearman's rank correlation owing to limitations in its testing range (2–8 mg/L). All Spearman values had a P value <0.0001.

Table 2. Performance of AST methods compared with reference BMD method using different resistance levels

AST method	CA, % (*n*)			EA, % (*n*)			VMD, % (*n*)		
	resistance level[a]			resistance level[a]			resistance level[a]		
	low ($n = 10$)	medium ($n = 18$)	high ($n = 3$)	low ($n = 10$)	medium ($n = 18$)	high ($n = 3$)	low ($n = 10$)	medium ($n = 18$)	high ($n = 3$)
Etest (24 h)[b]	20.0 (2)	27.8 (5)	100 (3)	10.0 (1)	5.6 (1)	NA	80.0 (8)	72.2 (13)	0.0 (0)
Etest (48 h)[b]	30.0 (3)	33.3 (6)	100 (3)	10.0 (1)	5.6 (1)	NA	70.0 (7)	66.7 (12)	0.0 (0)
MICRONAUT-S[b]	80.0 (8)	100 (18)	100 (3)	50.0 (5)	72.2 (13)	NA	20.0 (2)	0.0 (0)	0.0 (0)
SensiTest[c]	80.0 (8)	100 (18)	100 (3)	50.0 (5)	N/A	NA	20.0 (2)	0.0 (0)	0.0 (0)
Sensititre[b]	80.0 (8)	100 (18)	100 (3)	70.0 (7)	72.2 (13)	NA	20.0 (2)	0.0 (0)	0.0 (0)
Rapid PP[d]	90.0 (9)	100 (18)	100 (3)	NA	NA	NA	10.0 (1)	0.0 (0)	0.0 (0)

NA, not applicable.
[a]Resistance levels: low, 4–8 mg/L; medium, 16–64 mg/L; high, 128–512 mg/L.
[b]The Etest, MICRONAUT-S and Sensititre tests were excluded from the high-resistance division owing to the maximum MIC measurements being 256 mg/L, 64 mg/L and 128 mg/L, respectively.
[c]The SensiTest was excluded from the medium- and high-resistance divisions for EA, as the highest MIC that can be measured is 16 mg/L.
[d]The Rapid PP was excluded from EA calculations owing to the limited MIC range that it measures (2–8 mg/L).

Sensititre reporting the highest value (98%) when evaluated with susceptible isolates.

Discussion

We acquired 31 colistin-resistant strains with low effort owing to the culture automation in the morbidostat. This is a collection that might take even reference centres and large laboratories years to achieve, whereas in our case all strains attained various levels of resistance within 58 days. Besides colistin-resistant strains at various levels of resistance, we were furthermore able to select strains with MICs in the upper range of susceptible. This enabled us to measure the performance of AST methods in terms of categorization of isolates in all relevant MIC ranges.

Accurate colistin susceptibility testing methods are necessary to ensure an optimal patient outcome and to reduce the spread of resistance. Although all of the commercial AST methods except the Etest gained CA values >90%, none of them met the CLSI recommendations in all categories,[19] most notably for the critical VMD criterion. This was particularly the case for low-level colistin-resistant isolates, for which generally the test performance of all methods decreased significantly.

The Etest performed poorly compared with other AST methods, failing to reach the required CLSI minimum in three out of four categories (CA, EA and VMD), which may be owing to reduced diffusion of colistin through the agar. This pattern is replicated in other studies: in 2015, Dafopoulou *et al.*[20] found colistin Etests to have a VMD rate of 39.3% using *Klebsiella pneumoniae* and *Acinetobacter*

baumannii strains. A more recent study by Chew *et al.*[21] in 2017 found a VMD of 12%, measured with Enterobacteriaceae. In this study, the CA and EA of the Etest at 24 h were 84% and 70.5%, respectively, with a VMD rate of 67.7%.

Commercial BMD methods show stronger correlation with the reference BMD method compared with Etests. The Sensititre achieved the best results in our hands, meeting and exceeding the CLSI requirements in three out of four categories (CA, EA and MD). However, the VMD rate was high (6.5%). The Sensititre has been reported to have strong results in other studies with Gram-negative bacilli,[22–24] with Matuschek *et al.*[24] in particular reporting a 100% EA rate for both Sensititre and MICRONAUT-S tests; although their study included only 21 isolates of *P. aeruginosa*. Another study compared Sensititre with the reference BMD and found that it had a CA of 90.1% and EA of 89.5%, using strains of Enterobacteriaceae.[21] Their VMD rate was 4%, with one isolate out of 30 categorized incorrectly as susceptible.

It is important to note that the needs of the laboratory should be taken into account. Of the commercial BMD methods, the hands-on time for the Rapid PP was one of the shortest at 25 min to test one isolate with the shortest time to result (4 h), compared with 18–24 h for other AST methods, meaning that test results can be read on the same day of testing. However, the MICRONAUT-S has an MIC evaluation panel of up to 64 mg/L, making it appropriate for many laboratories and clinics, with a similar hands-on time.

Despite numerous studies reviewing commercial AST methods, it is difficult to generate any conclusions, as the reference methods used have varied, as well as the calculation methods for the categories.[22–26] A warning against gradient tests was issued by EUCAST in 2016,[6] and so only studies that use the recommended BMD method as a reference should be applied to clinical outcomes. This study uses the highest number to date of both susceptible and resistant *P. aeruginosa* isolates, a pathogen of critical status, in combination with colistin, a last-resort antibiotic. The results of the study shed light on the problem areas of AST methods, namely with isolates around the susceptibility breakpoint and at the lower end of the resistance scale.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S5 appear as Supplementary data at *JAC* Online.

References

1 Tacconelli E, Carrara E, Savoldi A *et al*. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* 2018; **18**: 318–27.

2 Tumbarello M, Repetto E, Trecarichi E *et al*. Multidrug-resistant *Pseudomonas aeruginosa* bloodstream infections: risk factors and mortality. *Epidemiol Infect* 2011; **139**: 1740–9.

3 Li J, Nation R, Turnidge J *et al*. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 2006; **6**: 589–601.

4 Kang C, Kim S, Kim H *et al*. *Pseudomonas aeruginosa* bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. *Clin Infect Dis* 2003; **37**: 745–51.

5 Fiaccadori E, Antonucci E, Morabito S *et al*. Colistin use in patients with reduced kidney function. *Am J Kidney Dis* 2016; **68**: 296–306.

6 EUCAST. *EUCAST Warnings Concerning Antimicrobial Susceptibility Testing Products or Procedures*. http://www.eucast.org/ast_of_bacteria/warnings/.

7 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically–Tenth Edition: Approved Standard M07-A10*. CLSI, Wayne, PA, USA, 2015.

8 Goldstein FW, Ly A, Kitzis MD. Comparison of Etest with agar dilution for testing the susceptibility of *Pseudomonas aeruginosa* and other multidrug-resistant bacteria to colistin. *J Antimicrob Chemother* 2007; **59**: 1039–40.

9 Galani I, Kontopidou F, Souli M *et al*. Colistin susceptibility testing by Etest and disk diffusion methods. *Int J Antimicrob Agents* 2008; **31**: 434–9.

10 Hawley J, Murray C, Jorgensen J. Colistin heteroresistance in *Acinetobacter* and its association with previous colistin therapy. *Antimicrob Agents Chemother* 2007; **52**: 351–2.

11 Dößelmann B, Willmann M, Steglich M *et al*. Rapid and consistent evolution of colistin resistance in extensively drug-resistant *Pseudomonas aeruginosa* during morbidostat culture. *Antimicrob Agents Chemother* 2017; **61**: e00043–17.

12 Magiorakos A, Srinivasan A, Carey R *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

13 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

14 *Spine: A Bioinformatics Tool for Pan-Genome Analysis|Comparative Genomics*. 2018. https://omictools.com/spine-tool.

15 Li H, Handsaker B, Wysoker A *et al*. The sequence alignment/map format and SAMtools. *Bioinformatics* 2009; **25**: 2078–9.

16 *MEGA — Molecular Evolutionary Genetics Analysis*. 2018. http://www.megasoftware.net/mega4/.

17 Toprak E, Veres A, Michel J *et al*. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet* 2011; **44**: 100–5.

18 *EUCAST Clinical Breakpoint Tables v. 8.0, Valid from 01/01/2018*. 2018. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.0_Breakpoint_Tables.pdf.

19 Clinical and Laboratory Standards Institute. *Verification of Commercial Microbial Identification and Antimicrobial Susceptibility Testing Systems–First Edition: M52-Ed1*. CLSI, Wayne, PA, USA, 2017.

20 Dafopoulou K, Zarkotou O, Dimitroulia E *et al*. Comparative evaluation of colistin susceptibility testing methods among carbapenem-nonsusceptible *Klebsiella pneumoniae* and *Acinetobacter baumannii* clinical isolates. *Antimicrob Agents Chemother* 2015; **59**: 4625–30.

21 Chew K, La M, Lin R *et al*. Colistin and polymyxin B susceptibility testing for carbapenem-resistant and mcr-positive Enterobacteriaceae: comparison of

Sensititre, MicroScan, Vitek 2, and Etest with broth microdilution. *J Clin Microbiol* 2017; **55**: 2609–16.

22 Hindler J, Humphries R. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol* 2013; **51**: 1678–84.

23 Jayol A, Nordmann P, André C *et al*. Evaluation of three broth microdilution systems to determine colistin susceptibility of Gram-negative bacilli. *J Antimicrob Chemother* 2018; **73**: 1272–8.

24 Matuschek E, Öhman J, Webster C *et al*. Antimicrobial susceptibility testing of colistin—evaluation of seven commercial MIC products against standard broth microdilution for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. *Clin Microbiol Infect* 2017; **24**: 865–70.

25 Giani T, Morosini MI, D'Andrea MM *et al*. Assessment of the Phoenix™ automated system and EUCAST breakpoints for antimicrobial susceptibility testing against isolates expressing clinically relevant resistance mechanisms. *Clin Microbiol Infect* 2012; **18**: E452–8.

26 Carretto E, Brovarone F, Russello G *et al*. Clinical validation of SensiTest Colistin, a broth microdilution-based method to evaluate colistin MICs. *J Clin Microbiol* 2018; **56**: e01523–17.