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The Evaluation of Four *In Vitro* Susceptibility Testing Methods for Colistin on Carbapenenm-Resistant *Acinetobacter baumannii*

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Abstract

Background: The carbapenem resistance in *Acinetobacter baumannii* (CRAB) is a global health problem because of the worldwide distribution of the bacteria and a few available therapeutic options. Colistin is considered as the last resort to treat the infection. At present, there are several methods to detect the colistin susceptibility, including broth microdilution with 0.002% polysorbate 80 (BMD-P80), the E-test, broth microdilution (BMD), and agar dilution (AD). However, the differences in efficacy between the methods are not well studied.

Objectives: The current study aimed at evaluating the 4 available methods to test *in vitro* susceptibility to colistin and observing the degree of heteroresistance in CRAB species in China.

Methods: To evaluate the methods, a total of 202 CRAB species isolated from 12 hospitals in Zhejiang province, China, collected from January to December in 2010 were employed retrospectively. Colistin minimum inhibitory concentrations (MICs) were determined by the 4 different methods. Population analysis profiles (PAPs) were also conducted in 29 CRAB strains.

Results: The proportions of colistin-sensitive isolates were 100%, 100%, 99.5%, and 90.6% by BMD-P80, E-test, BMD, and AD methods, respectively, according to the EUCAST breakpoints. The AD methods produced an excessive number of major errors (MEs) (9.4%), while E-test and BMD produced 0.5% MEs. Moreover, the AD method resulted in a minimum essential agreement (EA) at 9.4%, and 179 isolates obtained a higher ($\geq$ 3 log 2) number of dilutions than the BMD-P80. Not very major errors (VMEs) were found by any of the tested methods. In 29 selected CRAB isolates, a total of 31% were heterogeneous and the rate of heteroresistance was also 31%.

Conclusions: The AD method was not a prior choice of *in vitro* colistin susceptibility testing because it led to false colistin resistance results. E-test, BMD, and BMD-P80 may be more reliable to test the susceptibility when colistin is considered as a potential therapeutic agent. A high rate of heterogeneous and heteroresistant species were found in CRAB in China; it highlighted the importance of MICs monitoring before and through the colistin therapy period.

Keywords: Colistin Susceptibility, Population Analysis Profiles, *Acinetobacter baumannii*

1. Background

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is of great concern because of its wide ranging global diffusion. It is a major nosocomial pathogen and it is difficult to treat due to its very limited antibiotic options (1). Colistin is considered as the last resort to treat infections caused by CRAB isolates (2-4). Although the surveillance studies revealed that colistine sensitivity rates are fortunately very high, discovering the optimal colistin susceptibility test method remains a concern of clinical laboratories. The Clinical and Laboratory Standards Institute (CLSI) and the European committee on antimicrobial susceptibility testing (EUCAST) could not provide the disc diffusion method (DD) with the colistin breakpoints for *A. baumannii*. Because of the unreliable results obtained from the colistin DD tests, the manufacture of the colistin disks suggested that any methods yielding an minimum inhibitory concentration (MIC) should be used to confirm a DD zone for colistin within the susceptible range.

Nevertheless, the reliability of the colistin MICs remains a matter of concern (5). The MICs obtained by the E-test present a very major error range of 32%, as compared to those of the agar dilution (AD) (6, 7). Some studies used broth microdilutions (BMD) as the standard method to determine the colistin MICs. Colistin adheres to the plastics used for the BMD panels, affecting drug concentrations. Polysorbate 80 (e.g., Tween 80), a surfactant, mitigates the adsorption of colistin to polystyrene (8). However, the CLSI reference fails to stipulate the employment of a surfactant for colistin under the BMD method (9). Thus, it leads to a significant variability in the results imparted by the laboratories performing the BMD method according to the CLSI. The degree of discrepancy among these methods is

not well studied.

In addition, colistin is usually regarded as an empirical option to treat severe infections caused by CRAB species according to the *in vitro* susceptibility test result. However, heteroresistance to colistin has emerged in clinical isolates, including *A. baumannii* (4). The heteroresistance might lead to clinical failure and emergence of colistin resistance. Thus, the degree of colistin heteroresistance in CRAB species should be observed to provide an important reference in clinical usage.

2. Objectives

The current study aimed at evaluating the 4 available methods to test *in vitro* susceptibility to colistin and observing the degree of heteroresistance in CRAB isolates in China.

3. Methods

3.1. Bacterial Isolates

A total of 202 species of CRAB were studied in the current retrospective study. These isolates were obtained from patients hospitalized in 12 different hospitals in Zhejiang province, China, from January to December, 2010. All of the strains were assigned to *A. baumannii*, using Vitek GNI+ card (bioMe'rieux, Marcy-l'E'toile, France). Carbapenem resistance was confirmed by a broth microdilution, following the clinical and laboratory standard institute (CLSI) guidelines (9). The isolates were stored in glycerol stocks at -70°C until their use in the current study. The following isolates were used as controls in the susceptibility tests: *Escherichia coli* ATCC 25922 and *A. baumannii* ATCC 19606.

3.2. MIC of Colistin

3.2.1. Broth Microdilutions

Cation-adjusted Mueller-Hinton broth (OXOID, England) was used in the BMD test, according to the guidelines outlined in the CLSI reference (9). The colistin concentrations ranged from 0.03 to 64 μg/mL. The MIC was defined as the lowest concentration of colistin at which no visible growth was obtained.

3.2.2. Broth Microdilutions with Polysorbate 80

The test was performed according to the BMD method, as outlined in the above introduction. The final polysorbate 80 had a concentration in each well of 0.002%. The colistin concentrations ranged from 0.03 to 64 μg/mL. The MICs and breakpoints were defined according to the BMD method.

3.2.3. E-Test

The MIC of colistin was determined by the E-test method, according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

3.2.4. Agar Dilutions

The agar dilution (AD) method was performed according to the method detailed in the previous reports (6). Colistin powder (Sigma Aldrich, USA) and molten Mueller-Hinton agar (BD, USA) were used in the AD method. Colistin was prepared in 2-fold dilutions in concentrations ranging from 0.03 to 64 μg/mL. The bacterial inoculum was strictly adjusted according to the CLSI guidelines, using a nephelometer (BioMerieux, France), and was prepared simultaneously for each isolate in each of the 4 methods.

3.3. Interpretation of Susceptibility Results

The interpretation of the clinical resistance of colistin against *A. baumannii* varies due to its different MIC breakpoints, as set by CLSI (susceptible, MIC $\leq$ 2 mg/L; resistant and MIC $\geq$ 4 mg/L) (9) and EUCAST (susceptible, MIC $\leq$ 2 mg/L; resistant and MIC > 2 mg/L) (10). BMD-P80 was taken as the reference method in the current study. Essential agreement (EA) was defined as the percentage of the MICs, within $\pm 1 \log_2$ dilutions, as determined by BMD-P80. Categorical agreements (CAs) were defined by BMD-P80 and the method under evaluation as test results within the same susceptibility category. Error types were ranked as follows: very major errors (VME); false-susceptibility resulting from the BMD, AD and E-test; major errors (MEs), false-resistance results produced by the BMD, AD, and E-test; and minor error (mE), differences between the reference and test methods' differing by 1 interpretative category (11). Unacceptable levels were established at 3% for the VME and ME (12).

3.4. Heteroresistance and Heterogeneity

In the 29 selected isolates of CRAB, the heteroresistance and heterogeneity were determined by population analysis profile (PAP) according to the reported method (13). Briefly, solutions containing 7 distinct bacterial inoculums ranging from 10^2 to 10^8 CFU/mL were prepared. A 20-μL aliquot of each solution was spread on Mueller-Hinton agar plates containing 0, 0.5, 1, 2, 4, 6, and 8 mg/L of colistin. Colonies were counted following 48 hours of incubation at 35°C. Colistin MICs of these subpopulations growing at the highest colistin concentration were determined subsequent to 3 days of daily subculturing in antibiotic-free mediums. Heteroresistance was defined as subpopulations that grew on plates containing an excess of 2 μg/mL of colistin. Heterogeneity was defined as subpopulations that grew on plates containing colistin at concentrations of 2 $\times$ MICs and 2 μg/mL (14).

4. Results and Discussion

4.1. Contrasting Methods for the Determination of Colistin Against 202 Isolates of CRAB: MIC Distribution and Susceptibility

The BMD-P80 resulted in MIC_{50} at 0.125 mg/L and MIC_{90} at 0.5 mg/L, respectively. The E-test had an identical MIC_{90} to the BMD-P80. The BMD and AD acquired MIC_{90} of 2.0 mg/L, which were 2 gradients higher than those of the BMD-P80 and E-test. Significant differences also appeared in the MIC_{50}, at 0.38, 0.5, and 2 mg/L, when using the E-test, BMD, and AD, respectively. With reference to the EUCAST judgment standard, the BMD-P80, E-test, BMD, and AD for colistin susceptibilities were 100%, 100%, 99.5%, and 90.6%, respectively. With reference to the CLSI judgment standards, the BMD-P80, E-test, BMD, and AD for colistin susceptibilities were 100%, 100%, 99.5%, and 94.3%, respectively (Table 1). No isolates were found resistant to colistin when using the BMD-P80 and E-test, according to the breakpoints of both the CLSI and EUCAST, while the 0.5% isolates were resistant to colistin when using the BMD, according to the breakpoints of both the EUCAST and CLSI. However, 9.4% and 5.7% of the isolates were found resistant to colistin by the AD, according to the breakpoints of both the EUCAST and CLSI, respectively (Table 1).

4.2. The EA, CA, and the Types of Errors Produced by the BMD, AD, and E-Test, Compared with Those of the BMD-P80

Compared with the clolistin susceptibility results determined by the BMD-P80, the EA of the AD method in a minimum of 9.4% (19/202) and 179 isolates (88.6%) obtained higher (3 log2) dilutions than those with the BMD-P80. The EA of the E-test method in a maximum of 44.3% (89/202) and 160 isolates (79.2%) obtained higher (1 $\log_2$) dilutions than those following the BMD-P80. The EA of the BMD method was 38.5% (77/202) and 172 isolates (85.1%) obtained higher $\geq$ 1 $\log_2$ dilutions than the BMD-P80. Interestingly, the CA of the E-test, BMD, and AD, compared with those of the BMD-P80, were 99.5%, 99.5%, and 90.6%, respectively, according to both the EUCAST and CLSI. It was also discovered that, regardless of using the EUCAST or CLSI guidelines, no VME or ME existed among the BMD-P80, E-test, MD, or AD. Compared with the BMD-P80, the ME of the E-test, BMD, and AD were 0.5%, 0.5%, and 9.4%, respectively, according to both the EUCAST and CLSI guidelines (Table 2).

Acinetobacter baumannii is often spread, causing outbreaks in the hospitals, throughout the cities, countries, and continents (15). Colistin is the most important available antibiotic in China, which is effective against CRAB. Thus, accurate susceptibility testing of colistin remains critical. Among these 4 methods, the BMD is currently the only method recommended by the CLSI-EUCAST polymyxin breakpoints working group (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf). The BMD-P80 is always recommended by the CLSI to prevent the binding of lipoglycopeptides to plastics. When P-80 was added to the well in a final concentration of 0.002%, BMD testing revealed that the colistin MIC values were 4- to 8-folds lower than those of isolates in the absence of P-80 (16).

The current study results were similar to those of the previous studies. In the current study, the colistin MIC value determined by BMD-P80 was lower than that of BMD, E-test, and AD method. The results of E-test method were similar to those of the BMD. Compared with the BMD-P80, the CA of the E-test, BMD was almost 100%. The data of the current study also confirmed the limitation of AD method. Note that the AD method resulted in much higher MICs than BMD, obviously affecting the susceptibility and resistant rates. Employment of the AD method in clinical experiments resulted in a 9.4% false resistance. AD method appeared unreliable to detect colistin susceptibility in *A. baumannii*. In CRAB infected patients, the false colistin resistance might lead to a problematic situation with no apparent solution. E-test, BMD, and BMD-P80 may be more reliable for susceptibility testing when colistin is considered for use as a potential therapeutic agent. This may be the reason that the BMD remains the primary reference method to test colistin susceptibility by the joint CLSI-EUCAST polymyxin breakpoints working group.

4.3. A population Analysis Profile of the 29 Isolates of CRAB

Population analysis profile was performed to evaluate heterogeneity and heteroresistance in CRAB species. The PAP revealed the growth of subpopulations in all 29 isolates at an excess of 2-fold MICs of the original population. The colistin MICs of 31% (9/29) "higher MIC" subpopulations dropped to the same percentage of MICs of the original population, following the daily passages on the colistin-free medium, and 62% (18/29) of these isolates remained as subpopulations, growing at a 2 folds of the original concentration. Among the 18 isolates, a total of 9 isolates acquired subpopulations able to grow at higher colistin concentrations (> 2 mg/L), with the proportions of these subpopulations ranging from 2.5×10^{-7} to 6.2×10^{-4}. Interestingly, 2 isolates remained at a 4-fold MIC change following 3 days of daily passages on the colistin-free medium. In conclusion, 31% of the isolates were heterogeneous and 31% heteroresistant.

Colistin heteroresistance is described in *A. baumannii* worldwide, with a rate of 18.7% to 100% (17, 18). The current study confirmed a high rate of heterogenity and het-

Table 1. Colistin MICs Distribution and Susceptibility of the CRAB Species Using Different Methods

Method	MIC, mg/L			EUCAST, %		CLSI, %	
	Range	MIC_{50}	MIC_{90}	S	R	S	R
BMD	0.0625 - 4	0.5	2	99.5	0.5	99.5	0.5
BMD-P80	0.03 - 1	0.125	0.5	100.0	0.0	100.0	0.0
E-test	0.064 - 1.5	0.38	0.5	100.0	0.0	100.0	0.0
AD	0.0625 - 4	2	2	90.6	9.4	94.3	5.7

Abbreviations: S, Sensitive; R, Resistant.

Table 2. EA, CA, and Types of Errors Produced by BMD, AD, and E-test Compared with BMDP-80 on the CRAB Isolates (%)

Method	EA	EUCAST				CLSI			
		VME	ME	mE	CA	VME	ME	mE	CA
BMD	32.1	0.0	0.5	0.0	99.5	0.0	0.5	0.0	99.5
E-test	44.3	0.0	0.5	0.0	99.5	0.0	0.5	0.0	99.5
AD	9.4	0.0	9.4	0.0	90.6	0.0	9.4	0.0	90.6

eroresistance in CRAB species of China, most of which were susceptible to colistin on the basis of MICs (19). The heteroresistance might lead to clinical failure and emergence of colistin resistance, especially when inadequate dosing is administered. It highlighted the importance of optimizing the colistin regimen based on pharmacokinetics (PK)/pharmacodynamics (PD) according to exact MIC and MIC monitoring through the colistin therapy period.

5. Conclusion

In conclusion, it was found that the AD method leads to false colistin resistance results. The false resistance result potentially leads to inappropriate antibiotic administration and clinical failure. Therefore, the AD method may not be a prior choice of *in vitro* colistin susceptibility testing for *A. baumannii*. Besides, applying a proper susceptibility method before antibiotic prescription, and monitoring susceptibility are also important through the colistin therapy period because of a high rate of colistin heteroresistance in CRAB species.

Footnotes

Authors' Contribution: Contributed equally to this work, Hui Li, Hua Zhou; study design, Hui Li, Hua Zhou, and Yunsong Yu, experiments and tests conduction, Hui Li, Hua Zhou, Xi Li, and Jianfeng Wang; data analysis, Ying Fu and Yan Jiang; study supervision for the intellectual concepts, Hua Zhou and Yunsong Yu; writing of the manuscript, Hua Zhou.

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