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ORIGINAL ARTICLE

Detection of Colistin Susceptibility in Multi- drug Resistant *Pseudomonas Aeruginosa* and *Acinetobacter Baumannii* by four Different Methods

Amina Kandeel[1,2,] PhD*

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Egypt
[2]Microbiology Department, King Khaled general Hospital, Hafer Albatin, Saudi Arabia

Key words:

Colistin resistance, Pseudomonas aeruginosa, Acinetobacter baumannii, E test, microscan, Disc diffusion, Agar dilution

ABSTRACT

Background: *Considering the increasing use of colistin for the treatment of serious infections and the emergence of resistance to this antibiotic in some countries, accurate and reliable susceptibility testing methods are essential.* ***Objectives:*** *evaluation of colistin susceptibility of multi- drug resistant P. aeruginosa and A.baumannii using four different methods.* ***Methodology:*** *All multi- drug resistant Pseudomonas aeruginosa (P. aeruginosa) and Acinetobacter baumannii (A. baumannii) isolated between June 2013 to May 2014 were included in the study. They were identified by Microscan walkaway96 system. Colistin susceptibility testing was done by agar dilution as a reference method, disc diffusion, E test in addition to the result obtained by Microscan.* ***Results:*** *In all multi- drug resistant P. aeruginosa and A. baumannii (72%,58%) were isolated from ICU patients respectively. The lower respiratory tract samples were the main source of both organisms (57.2%). Colistin showed activity against 97%(35/36) of A. baumannii and 92% (210/228) of P. aeruginosa by agar dilution method. The categorical agreement of Microscan with agar dilution was 97% for A. baumannii and 98% for P. aeruginosa. Poor categorical agreement (92.5% , 94%) was detected between disc diffusion and agar dilution for P. aeruginosa and A. baumannii respectively whereas excellent categorical agreement was found between E test and the reference method(99.6% and 100%) for both organisms respectively.* ***Conclusions:*** *E test and Microscan are reliable methods to test colistin susceptibility in multi- drug resistant P. aeruginosa and A. baumannii while disc diffusion results are inaccurate and need to be confirmed by another method.*

INTRODUCTION

Colistin is a cationic polypeptide antibiotic which was used for treating infections caused by Gram negative bacilli till the early 1980s. Its systemic use was discontinued because of problems, such as nephrotoxicity, neuromuscular blockade, and neurotoxicity[1,2]. However, the emergence of bacteria resistant to most classes of commercially available antibiotics and the shortage of new antimicrobial agents have led to the reconsideration of colistin as a valuable therapeutic option.[3] Infections caused by *Pseudomonas aeruginosa (P. aeruginosa*) and *Acinetobacter baumannii (A. baumannii*) are challenging to treat due to their resistance to multiple antibiotics [4-6].

Corresponding Author:
Amina Kandeel
Microbiology Department, King Khaled general Hospital, Hafer Albatin, Saudi Arabia.
E-mail address: amina_449@yahoo.com;
Tel.: +966553109967, fax: +966 013 7213808.

Therefore they are among the main pathogens targeted by colistin and the Clinical and Laboratory Standards Institute (CLSI) has published minimum inhibitory concentration (MIC) interpretation guidelines only for these organisms.[7] Currently, colistin resistance is being reported all over the world. Among the colistin-resistant bacteria, *A. baumannii* and *P. aeruginosa* are the most common[8].

Considering the increasing use of colistin for the treatment of serious infections and the emergence of resistance to this antibiotic in some countries, accurate and reliable susceptibility testing methods are essential [9]. The dilution methods remain the gold standard, but they are difficult to perform as routine tests in many clinical laboratories. Some studies have reported good concordance between agar dilution and micro broth dilution [9,10] Disk diffusion is a commonly used method for measuring colistin susceptibility. But as colistin diffuses poorly in agar, it produces small inhibition zones resulting in poor differentiation of susceptible and resistant isolates[11].

On the other hand, excellent correlations between the E test and the broth microdilution and agar dilution tests were demonstrated, suggesting that these methods, rather than disk diffusion methods, should be used to determine susceptibility to colistin. As regard to the performance of automated antimicrobial susceptibility testing systems while Lo-Ten-Foe et al., showed a high level of agreement between Vitek2 and the reference method , Tan and Ng reported that it was unreliable for detecting colistin resistance in Gram-negative bacilli[12,13]. The performance of MicroScan for colistin susceptibility testing has not been reported except in one study in which the authors concluded that the MicroScan was unsuitable for colistin susceptibility testing of *Acinetobacter* species, due to its low reliability[14].The aim of the present study is to evaluate colistin susceptibility of multi- drug resistant P. aeruginosa and A.baumannii using four different methods namely, Microscan WalkAway 96,E test and disk diffusion in comparison with the agar dilution method

METHODOLOGY

1. Study design

This prospective study was carried out at King Khaled general hospital eastern province, Saudi Arabia from June 2013 to May 2014. All multi- drug resistant (MDR) *P. aeruginosa* and *A. baumannii* isolated from different samples submitted to microbiology laboratory were included in the study. Duplicate isolates from the same patient were considered as one. Clinical data including age, sex and location of admission were recorded from microbiology request data.

2. Microbiological methods

The bacterial identifications and antibiotic sensitivity tests were performed by MicroScan WalkAway 96 system (Siemens, Sacramento, USA) using negative breakpoint combo42(NBC42) panels with interpretation of results by Microscan software program, according to the guidelines of the CLSI [7]. The isolates were considered MDR if they were resistant to 3 or more classes of antibiotics used for treatment of these infections. Colistin sensitivity in the Microscan system was evaluated by the broth microdilution method with MIC ranging from 2 to 4 µg/ml.

a. E test

The E-test (bioMérieux, France) was performed for the determination of MIC values. For the E-test method, the bacterial suspension, which was calibrated to 0.5 McFarland opacity, was cultivated onto Mueller–Hinton agar (MHA) (Bio-Rad) in accordance with the manufacturer's recommendations, after which the E-test colistin strip (ranging from 0.016 to 256 µg/ml) was positioned. MIC values were determined after 16–20 hour of incubation at 35°C. The susceptibility test results were interpreted according to CLSI breakpoint recommendations in the E-test and broth microdilution methods, ≤2 mg/l and ≥4 mg/l were accepted as sensitive and resistant, respectively for *A. baumanii* . For *P. aeroginosa*, ≤2 mg/l, 4 mg/l and ≥8 mg/l were accepted as sensitive, intermediate and resistant respectively.[7]

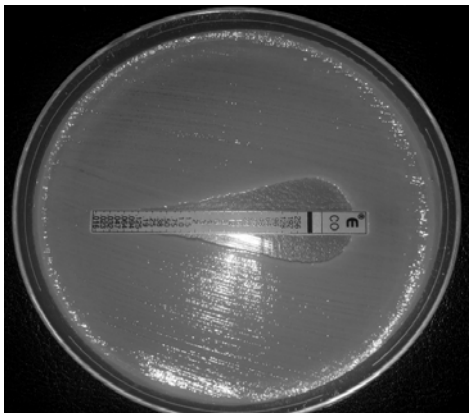

Fig. 1: Showing *P. aeroginosa* isolate sensitive to colistin by E test

b. Agar dilution method (reference method)

MIC of colistin were obtained by the agar dilution method. Colistin sulfate powder (Sigma-Aldrich, Germany) was dissolved in sterile water and added to molten

MHA to provide twofold concentrations ranging from 0.25 to 64 mg/ l. Bacterial suspensions were adjusted to 0.5 McFarland (contain 10^8 colony-forming units (CFU)/mL) then diluted 1:10 in saline to obtain a concentration of 10^7CFU/ml. They were applied to agar plates to yield a final inoculum of 10^4 CFU per spot. Results were read after incubation at 35ºC for 16-20 h. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth.[7]

c. Disk diffusion method

Bacterial suspensions were adjusted to a turbidity equivalent to 0.5 McFarland standard prior to inoculation onto MHA. 10-µg colistin (Bio-Rad) discs were used. Zone diameter of ≥11 mm and ≤10 mm were interpreted as sensitive and resistant, respectively for P. aeroginosa[15]. For *A. baumanii*, ≤11 mm and ≥14 mm were interpreted as resistant and susceptible respectively[16].

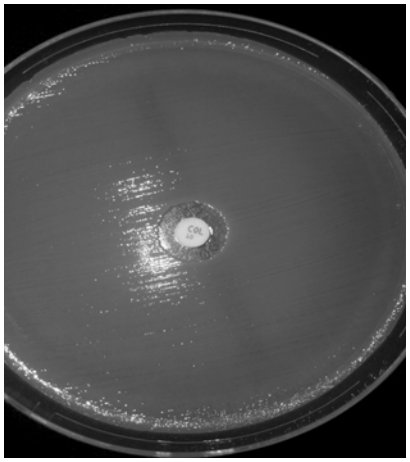

Fig. 2: Showing *A. baumanii* isolate resistant to colistin by disc diffusion method

3. Statistical analysis

Categorical agreement (CA) was defined as the percentage of isolates classified into the same category by the reference method (agar dilution method) and the test method. Errors were ranked as follows:

1. Very major error(vmj): if the result of the agar dilution was resistant(R), while that of the test method was sensitive(S) (false-susceptible result);
2. Major error (mj): if the result of agar dilution was S, while that of the test method was R (false-resistant result); and
3. Minor errors (mn): if agar dilution is intermediate (I) and test method is R or S or test method is I and agar dilution is S or R.

Unacceptable levels were greater than 1.5% for vmj, >3% for mj and 10% for mn..

RESULTS

Over 12 months**,** 228 MDR *P. aeruginosa* and 36 MDR *A. baumannii* were isolated from different wards. Of note, 60% of these isolates were obtained from ICU(table 1). The majority of isolates were from respiratory samples (57.2%), as shown in table 2. As shown in table 3 colistin had the highest sensitivity against both MDR *P. aeruginosa* and *A. baumannii* followed by tigecycline for *A. baumannii* and Piperacillin-tazobactam for *P. aeruginosa*. E-test produced similar colistin susceptibility results with agar dilution with (99.6%) CA for *P. aeruginosa* and 100% for *A. baumannii* whereas, Microscan produced lower rates of CA 98% and 97% for both organisms respectively. No vmj were reported for both methods (table 4). Comparison of disc diffusion with agar dilution demonstrated (92.5%), (94%) CA with high rates of vmj (4%), (2.8%) for *P. aeruginosa* and *A. baumannii* respectively (table 5).

Table 1: Distribution of *P. aeruginosa and A. baumannii* in different wards

Ward	**P. aeruginosa**		**A. baumannii**		***Total***	
	No	***%***	***No***	***%***	***No***	***%***
ICU	132	58%	26	72%	158	60%
MMW	32	14%	6	16.8%	38	14%
MSW	19	8.3%	2	5.6%	21	8%
FMW	21	9.2%	0	0%	21	8%
FSW	13	5.7%	0	0%	13	5%
Other wards	11	4.8%	2	5.6%	13	5%
Total	228	100%	36	100%	264	100%

FMW female medical ward; FSW: female surgical ward; ICU : intensive care unit ; MMW: male medical ward; MSW: male surgical ward.

Table 2: Distribution of *P.aeruginosa* and *A. baumannii* in different samples

sample	**P. aeruginosa**		**A. baumannii**		***Total***	
	No	**%**	***No***	***%***		
*Respiratory	120	52.6%	31	86%	151	57.2%
Wound swabs	46	20.2%	2	5.6%	48	18.2%
Blood	14	6.1%	1	2.8%	15	5.6%
Urine	48	21.1%	2	5.6%	50	19%
Total	228	100%	36	100%	264	100%

*Respiratory samples, included sputum, bronchoalveolar lavage and tracheal aspirates.

Table (3): Antibiotic susceptibilities of *P. aeruginosa* and *A. baumannii* by microscan

Antibiotic	P. aeruginosa		A. baumannii	
	No	%	No	%
Gentamicin	121	53%	34	12%
Amikacin	137	60%	26	9%
Tobramycin	124	54%	26	9%
Cefepime	112	49%	11	4%
Ceftazidime	110	48%	7	19%
Ciprofloxacin	121	53%	17	6%
Imipenem	128	56%	11	4%
Meropenem	132	58%	11	4%
Piperacillin	128	56%	11	4%
Piperacillin-tazobactam	153	67%	31	11%
Tigecycline	46	20%	86	31%
Colistin	205	90%	94	34%

Table 4: Comparison of agar dilution with E-test and Microscan MIC values for colistin susceptibility

Organism	*Test method*	*colistin MIC(µg/ml)*			*No. (%) of isolates*			*% CA*	*% of category Error*		
		≤2	*4*	*≥ 8*	*S*	*I*	*R*		*vmj*	*mj*	*mn*
P. aeruginosa	Agar D.	210	8	10	**210**	**8**	**10**				
	Micoscan	205	10	13	**205**	10	13	98%		3(1.3%)	2(1%)
	E test	209	8	11	209	**8**	**11**	99.6%		1(.4%)	
A. baumannii	Agar D.	35		1	35		1				
	Micoscan	34	1	1	34		2	97%		1(2.8%)	
	E test	35		1	35		1	100%			

Agar dilution : Agar D. The colistin MIC ranges determined by MicroScan were ≤2 and >4 µg /ml and were presented as 2,4 and 8 µg /ml, respectively. For MIC in Agar D and E test , all values less than or equal 2 are represented in as ≤2 while all values more than or equal 8 are represented as ≥ 8

Table 5: Comparison of disk diffusion zone diameters and agar dilution MIC values for colistin susceptibility

Organism	*Disc diffusion*	*Agar D values (µg/ml)*			*Total*	*% CA*	*% of category error*		
		≤2	*4*	*≥ 8*			*vmj*	*mj*	*mn*
P. aeruginosa	≤10 (R)		8	**1**	9				8(3.5%)
	≥11(S)	**210**		9	219	92.5 %	9(4%)		
	Total	210	8	10	228				
A. baumannii	≤11 (R)		**1**		1			1(2.8%)	
	≥14(S)	**34**	**1**		35	94%	1(2.8%)		
	Total	**35**	**1**		36				

DISCUSSION

The increasing prevalence of MDR nosocomial pathogens such as *A baumannii* and *P aeruginosa* poses a great challenge to the treating physicians. Due to the steady increase in bacterial resistance, the use of colistin is increasing which demands accurate and reliable in vitro susceptibility testing methods.[8]

In the present study 85% MDR *P. aeruginosa* and 72% *A. baumannii* were isolated from ICU patients. This is similar to findings in other studies [17-19].This is due to its extremely vulnerable population ,increased risk of infection through use of invasive devices and administration of several drugs which predispose to infections.[20]

The lower respiratory tract samples were the main source of both organisms (57.2%) which is similar to other studies [21]. Rit, et al reported that *Acinetobacter spp* and *P. aeruginosa* were the most common pathogens causing late onset ventilator associated pneumonia (VAP)[22]. Also Dey and Bairy study showed *A.baumannii*, *P.aeruginosa* were most common isolates for both early & late onset VAP[23].

In the current study colistin demonstrated the highest activity against *A. baumannii* and *P. aeruginosa* isolates as 97% and 92% were sensitive respectively. This result is in concordance with other reports which

stated that colistin had excellent bactericidal activity against most gram-negative aerobic bacilli, including *Acinetobacter* species, *P. aeruginosa* [24,25,3].In Kuwait Sweih et al.[26] reported colistin resistance in *A. baumannii* to be 12%. As regard to *P. aeroginosa* Ateba et al. reported 98% retained sensitivity to colistin.

Among MDR *P. aeruginosa* piperacillin-tazobactam had good activity (67%) followed by amikacin(60%) whereas tigecycline showed the lowest activity 20% as tigecycline has limited activity against *P. aeruginosa*, [28,29]

In the present study tigecycline demonstrated good activity against *A. baumannii* (85%). Somily et al.[30] found that 89.3% of *A. baumannii* strains were susceptible to tigecycline while only 15.2% of *P. aeruginosa* were sensitive.21 In a surveillance study from Germany , tigecycline resistance among *A. baumannii* isolates was 6%, whereas, colistin resistance was 2.8%.

In our study, poor CA(92.5%,94%) was detected between disc diffusion and agar dilution with(4%) and (2.8%) vmj for *P. aeruginosa* and *A. baumannii* respectively. Other studies comparing disc diffusion test for polymyxins with reference method have consistently reported it to be unreliable for use as polymyxins are large molecules and diffuse poorly into the medium to produce inconsistent inhibition zones [31,19]**.** However Sinirtaş et al. found 100% CA between the disk diffusion and E test when tested against *A. baumannii* but no isolates were resistant to colistin in that study.[18]

For the performance of automated systems Lo-Ten-Foe et al.[12] compared the Vitek 2and broth microdilution methods for colistin susceptibility and found a high level of agreement and concluded that it can be considered as a reliable tool to determine susceptibility to colistin in isolates of genera that are known not to exhibit resistant subpopulations. This has been contradicted by Tan and Ng.b[13] who have deemed Vitek 2 to be an unreliable method with unacceptable rates of vmj (18%). Sinirtaş et al.[12] concluded in their study that 100% CA exists between the Phoenix system and the E-test.

Micoscan performance was reported in one study in which the CA with agar dilution was 95.7% for *A. baumannii* but 80.7% for non-*baumannii Acinetobacter* isolates. The authors suggested that non-*baumannii Acinetobacter* species were the main source of errors. They explained their result by narrow distribution of colistin MICs (2 to 4 µg /ml), compared with E test (0.016 to 256 µg g/ml) or Vitek 2 (≤0.5 to≥16 µg g/ml).[14]

In another study evaluating colistin among KPC-producing *Klebsiella pneumoniae,* the authors reported that Microscan automated system did not seem to be very efficient for the screening of polymyxin-resistant isolates once an inappropriate sensitivity is achieved.[3]

In our study, which is considered the third one to report the performance of Micoscan the CA of it with agar dilution was 97% for *A. baumannii* and 98% for *P. aeruginosa.* It produced one mj in A. *baumannii* and three mj and one mn error in *P. aeruginosa*.

Among commercial methods, E test is convenient and widely applied in clinical laboratories. [33] Most studies have demonstrated the concordance of the E-test to be as high as 90 - 100% and have suggested it as a reliable and useful alternative to the dilution methods [34,35]. While Lo-Ten-Foe et al.[12] showed that disk diffusion method to be unreliable in their study, they reported a high level of agreement between the E test and broth microdilution method. Another study comparing colistin E tests with broth microdilution for *A. baumannii* reported a vmj error rate of 1.7% [36].

On the other hand when Tan and Ng compared the E-test with agar dilution for colistin susceptibility, they reported 5(11%) mj and 14(30%) vmj when testing P. aeruginosa, and one error(2%) when testing *Acinetobacter* spp. They concluded that results obtained by E test may require confirmation by a standard MIC susceptibility testing method [13]. In our study, E test showed100% CA with reference method in *A. baumannii* and 99.6% in *P. aeruginosa*

Conflict of interest statement
None declared.
Funding source
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