

Comparison of E-test and disc diffusion methods for the in vitro evaluation of the antimicrobial activity of colistin in multi-drug resistant Gram-negative Bacilli

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ABSTRACT

الأهداف: دراسة حساسية العزلات السريرية من البكتيريا السالبة لصبغة غرام ذات المقاومة للعقاقير المتعددة للكوليسيتين (MDR) بتحديد (MIC) باستخدام اختبار E ومقارنة هذا باختبار الحساسية بطريقة انتشار القرص (DD).

الطريقة: لقد تم اختبار عدد 224 عزلة من البكتيريا السالبة الصبغة غرام ذات مقاومة لعقاقير متعددة (147 عصيات الراكدة، و49 أنواع من الراكدة، و24 ستينوتروفوموناس مالتوفيليا، و43 سيودوميناس ايروجينوسا) خلال الفترة من يناير 2007م حتى أغسطس 2008م - مستشفى جامعة الملك خالد - الرياض - المملكة العربية السعودية. كما أنه قد تم تحديدها بأساليب حيوية معيارية. هذا وقد تم تفسير الحساسية بطريقة انتشار القرص DD و MIC باختبار E وفقا للنقاط الحاسمة للحساسية لمعهد المعايير السريرية والمعملية.

النتائج: باستخدام طريقة MIC، تم اكتشاف أن الكوليسيتين نشيط ضد 100% من أنواع الراكدة، و98% العصيات، و84% سيودوميناس ايروجينوسا، و79% ستينوتروفوموناس مالتوفيليا. كان نظام MIC⁹⁰ التصاعدي للكوليسيتين هو 1 مليجرام للعصيات الراكدة، و1.5 مليجرام من أنواع الراكدة، و3 مليجرام سيودوميناس ايروجينوسا، و16 ستينوتروفوموناس مالتوفيليا. وبمقارنة طريقة انتشار القرص بطريقة اختبار E وجدت أخطاء كبيرة بنسبة 1.4% للعصيات الراكدة و2.3% لستينوتروفوموناس مالتوفيليا، وأخطاء قليلة ثانوية بنسبة 0.7% للعصيات الراكدة، و8.3% لستينوتروفوموناس مالتوفيليا، و11.6% لوسيدوميناس ايروجينوسا.

خاتمة: أن الكوليسيتين نشيط ضد معظم البكتيريا السالبة لصبغة غرام متعددة MDR المقاومة للعقاقير التي تم اختبارها. هذا وتظهر طريقة انتشار القرص أخطاء قليلة ثانوية هامة عند مقارنتها بطريقة اختبار E. نوصي باستخدام طريقة MIC لاختبار حساسية البكتيريا العسوية السالبة لصبغة غرام المقاومة للعقاقير المتعددة للكوليسيتين.

Objectives: To study susceptibility of clinical isolates of multi-drug resistant (MDR) Gram-negative bacilli to colistin by minimum inhibitory concentration (MIC) determination using Etest, and compare this with their susceptibility measured by the disc diffusion (DD) method.

Methods: A total of 224 of MDR organisms (147 *Acinetobacter baumannii* [*A. baumannii*], 49 *Acinetobacter species*, 24 *Stenotrophomonas maltophilia* [*S. maltophilia*], and 43 *Pseudomonas aeruginosa* [*P. aeruginosa*]) were tested between January 2007 and August 2008 at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia. They were identified by standard microbiological methods. Susceptibility by DD and MIC by Etest were interpreted according to the Clinical and Laboratory Standards Institute breakpoints.

Results: Using the MIC method, colistin was found to be active against 100% of *Acinetobacter species*, 98% of *A. baumannii*, 84% of *P. aeruginosa*, and 79% of *S. maltophilia*. An ascending order MIC⁹⁰ of colistin was 1 µg/ml for *A. baumannii*, 1.5 µg/ml for *Acinetobacter species*, 3 µg/ml for *P. aeruginosa*, and 16 µg/ml for *S. maltophilia*. Comparing DD with the Etest method, very major errors of 1.4% were found for *A. baumannii* and 2.3% for *P. aeruginosa*, with minor errors of 0.7% for *A. baumannii*, 8.3% for *S. maltophilia*, and 11.6% for *P. aeruginosa*.

Conclusion: Colistin was active against most of the MDR isolates tested. The DD method showed significant errors when compared with the Etest method. We recommend using the MIC method to test the susceptibility of MDR Gram-negative bacilli organisms to colistin.

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Colistin (polymyxin E) is a polypeptide antibiotic belonging to the polymyxin group produced by *Bacillus polymyxa*.¹ Like other polymyxins, colistin is inactive against Gram-positive organisms, and the systemic administration of polymyxin was initially used in Japan and Europe during the 1950s and later in the United States in the form of colistimethate sodium in 1959.² However, the intravenous formulations of colistin and polymyxin B were gradually abandoned in most parts of the world in the early 1980s because of high reported incidences of nephrotoxicity.³ Over the past 2 decades, intravenous use of colistin was limited to the treatment of lung infections due to multidrug-resistant (MDR) Gram-negative bacteria in patients with cystic fibrosis.⁴ The drug has been increasingly used to treat infections caused by Gram-negative organisms susceptible only to this agent.⁵ Currently, there are conflicting recommendations on breakpoint interpretation from the Clinical and Laboratory Standard Institute (CLSI) issued initially in 2005, the British Society for Antimicrobial and Chemotherapy (BSAC), and the Societe Francaise de Microbiologie (SFM).⁶ Due to the limited pharmacokinetic and pharmacodynamic data, susceptibility testing for colistin remains problematic. Since the use of colistin is not widespread, it is unclear which breakpoint is most appropriate for interpretation of susceptibility to colistin.^{6,7} Our aim for this study was to evaluate the susceptibility of clinical significant isolates of Gram-negative bacilli to colistin using the minimal inhibitory concentration (MIC) susceptibility testing method and the disc diffusion (DD) comparative method.

Methods. This prospective study was conducted at King Khalid University Hospital (KKUH) in Riyadh, Kingdom of Saudi Arabia. This hospital has a capacity of 850 beds with primary, secondary, and tertiary care, serving a population of approximately 2 million. The study was approved by the hospital ethics committee. Susceptibility testing was conducted on all MDR Gram-negative bacilli strains isolated from different clinical specimens (respiratory, blood, body fluid, tissues, and urine) submitted to the microbiology laboratory over 32 months from January 2007 to August 2008. Patients' clinical data (including age, gender, ICU or non-ICU location, and specimen type) were collected from the laboratory request forms. Multiple isolates from the same patient were counted as one. Thirty-nine patients were infected with more than one organism, and of these 33 were infected with 2 organisms, 4 were infected with 3 organisms, and 2 patients were with infected with 4 organisms. A total of 42 patients were infected in more than one site. Of these, 21 were infected in 2 sites, 15 in 3 sites, 4 were infected in 4 sites, while 2 were infected

in 5 sites. The organisms were identified using API E (bioMerieux, Marcy-L'Etoile, France) and/or MicroScan (MicroScan Walk Away System 96, Dade Behring Inc, a Siemens Company, West Sacramento, CA, USA). The colonies were inoculated into MicroScan Dried Gram-negative Combo Panel Type 30 and Gram-negative Combo Panel Type 34. Isolates selected for inclusion in the study were considered MDR organisms using susceptibility profiles (namely, if these were resistant to 3 or more classes of antibiotics used for treatment of these infections). The MIC to colistin were conducted using Etest (AB Biodisk Solna, Sweden), and the interpretation of susceptibility was completed according to the CLSI breakpoint guideline, considering MIC ≤ 2 $\mu\text{g/ml}$ as susceptible, 2- ≤ 4 $\mu\text{g/ml}$ as intermediate, and 4- ≤ 8 $\mu\text{g/ml}$ as resistant to *Pseudomonas aeruginosa* (*P. aeruginosa*). The corresponding susceptible and resistant MIC breakpoints for *Acinetobacter spp.* were ≤ 2 $\mu\text{g/ml}$ and ≥ 4 $\mu\text{g/ml}$. For *Stenotrophomonas maltophilia* (*S. maltophilia*), the antimicrobial testing was interpreted using non-Enterobacteriaceae, and isolates with MIC ≥ 8 $\mu\text{g/ml}$ were considered as resistant. These isolates were tested by the DD comparative method following the CLSI recommendation using 10 μg colistin sulfate disk (Oxoid) and Mueller-Hinton agar. This was completed using a solution of bacterium with a 0.5 McFarland standard for inoculum inhibition followed by a 16-18 hour incubation. The isolate was considered susceptible if the zone of inhibition was ≥ 11 mm and resistant if the zone was ≤ 10 mm.⁸ *Pseudomonas aeruginosa* (ATCC strain 27853) and *Escherichia coli* (ATCC strain 25922) were used as quality control strains. Categorical agreement was defined if these results were within the same susceptibility category, as determined by the method in the CLSI (formally called NCCLS) M23-A2 and ranked as follows: 1) very major error, false-susceptible result by the DD test; 2) major error, false-resistant result produced by the DD test; and 3) minor error, intermediate result by the DD method and a resistant or susceptible category by the Etest.⁹ As with any clinical laboratory, the microdilution method is not readily available and needs validation, therefore, this method was not used as a reference method to evaluate results of Etest and DD.

Data were analyzed using the Statistical Package for Social Sciences Version 12.0 (SPSS Inc., Chicago, IL, USA) to calculate the correlation of susceptibility pattern and the error type of the susceptibility of isolates tested by the MIC method (Etest) and DD method. *Enterobacteriaceae* were excluded from the analysis because there are no specific CLSI breakpoints for these bacteria with this drug, and an insignificant number of strains exist to properly test resistance.

Results. Demographic information. Over 32 months, we collected a total of 273 clinical isolates that were classified as MDR organisms from 224 patients (Table 1). Two-thirds of the patients were male, and most of them were adult patients. More than half of the bacterial strains were isolated from patients in ICUs. *Acinetobacter* spp., mainly *Acinetobacter baumannii* (*A. baumannii*), was the most common isolate followed by *P. aeruginosa*, *S. maltophilia*, and *Burkholderia cepacia* (*B. cepacia*). These strains were most commonly obtained

Table 1 - Patient demographic details (N=224).

Variable	Number of patients (%)	
Gender		
Male	142	(63.4)
Female	82	(36.6)
Age		
≤1 year	15	(6.7)
>1-20 years	26	(11.6)
>20-50 years	87	(38.8)
>50 years	96	(42.8)
Location		
Outpatient	13	(5.8)
Emergency room	8	(3.6)
Inpatient	92	(41.1)
Intensive care units	111	(45.9)
Medical	54	(23.1)
Surgical	35	(15.6)
Coronary	10	(4.5)
Neonatal	8	(3.6)
Pediatric	4	(1.8)

from respiratory sites, followed by wounds, swabs, and urine. A total of 273 clinical specimens were obtained from these patients. One hundred and thirty (47.6%) were respiratory, 60 (21.9%) were wound swab, and 30 (10.9%) were surface swabs. While urine comprise of 20 (7.3%) specimens, catheter tips 13 (4.8%), body fluids 12 (4.4%), blood 5 (1.8%), and tissues 3 (1.1%). Of the total 273 organisms isolated, 147 (53.8%) were *A. baumannii*, 49 (17.9%) were *Acinetobacter* spp., 43 (15.7%) were *P. aeruginosa*, and 24 (8.8%) were *S. maltophilia*. Two specimens each were *B. cepacia* and *Enterobacter* spp. The rest was one strain each of *Serratia marcescens*, *Enterobacter freundii*, *Aeromonas hydrophila*, *Raoultella* spp., and *Proteus mirabilis*.

Antimicrobial activity. The antimicrobial activity of colistin against MDR Gram-negative nosocomial isolates is summarized in Table 2. Colistin exhibited the highest potency against *A. baumannii* and *Acinetobacter* spp. Due to the known toxicity of colistin, it was used on those patients where isolates were susceptible only to this agent. Most of these patients were in the ICUs and have other non-infectious confounding factors affecting their response to the drugs. This makes addressing the issue of correlation between the clinical response and the laboratory results difficult with the available data.

Comparison of the DD and MIC methods. Table 3 illustrates the discordant results between the DD and MIC methods, presented according to the error category of colistin testing. These results indicate very

Table 2 - Activity of colistin against multidrug resistant Gram-negative organisms shown as minimum inhibitory concentration (MIC) susceptibility (range 50 and 90) and susceptibility by the disc diffusion (DD) method.

Bacterium (n)	MIC range	MIC ⁵⁰ (µg/ml)	MIC ⁹⁰ (µg/ml)	No. of strains (%)	
				Susceptibility by Etest	Susceptibility by DD
<i>Acinetobacter baumannii</i> (n=147)	0.04 - 4	0.5	1.0	144 (98)	147 (100)
<i>Acinetobacter</i> spp. (n=49)	0.1 - 2	1.0	1.5	49 (100)	49 (100)
<i>Pseudomonas aeruginosa</i> (n=43)	0.13 - 24	1.5	3.0	36 (83.7)	42 (97.7)
<i>Stenotrophomonas maltophilia</i> (n=24)	0.19 - 256	0.38	16.0	19 (79.2)	20 (83.3)
<i>Burkholderia cepacia</i> (n=2)	0.75 - 3	0.75	3.0	1 (50)	2 (100)

Table 3 - Discordant results between the susceptibility methods (listed by number and category of error) for each organism tested by disc diffusion (DD) and minimum inhibitory concentration (MIC) by Etest.

Bacterium (n)	DD versus MIC n (%)		
	Very major error	Major error	Minor error
<i>Acinetobacter baumannii</i> (n=147)	2 (1.4)	0	1 (0.7)
<i>Acinetobacter</i> spp. (n=49)	0	0	0
<i>Pseudomonas aeruginosa</i> (n=43)	1 (2.3)	0	5 (11.6)
<i>Stenotrophomonas maltophilia</i> (n=24)	0	0	2 (8.3)
<i>Burkholderia cepacia</i> (n=2)	0	0	1 (50)*
Total (n=265)	3 (1.1)	0	3 (1.1)

NS - not significant due to small number of isolates

major error rates for *A. baumannii* and *P. aeruginosa*. Additionally, minor error rates were found for *A. baumannii*, *P. aeruginosa*, and *S. maltophilia*. No major errors were detected for *S. maltophilia*. Correlation was 100% for *Acinetobacter spp.*, meaning that all strains were susceptible by both methods. Only w strains of *B. cepacia* were isolated from these clinical specimens. This number was too small to draw conclusion from comparing susceptibility by disc diffusion and E-test method.

Discussion. The emergence of MDR non-fermentative Gram-negative bacteria in nosocomial infections has presented a medical challenge worldwide over the past decade.¹⁰ This has renewed interest in colistin, a selectively effective bactericidal agent against Gram-negative bacteria with no activity against Gram-positive organisms. Physicians are hesitant to use it due to its increased risk of toxicity (mainly nephrotoxicity) and narrow spectrum of action.¹¹ Recent studies have shown the effectiveness of colistin when compared with commonly used antimicrobial alternatives, such as carbapenems.¹² The side effects of permanent kidney damage of colistin can be reduced if patients are monitored for kidney function and serum drug level over the duration of antimicrobial therapy.¹⁴ According to published data,^{15,16} colistin should perform as a bactericidal agent against *Acinetobacter spp.*, *P. aeruginosa*, and most members of the *Enterobacteriaceae* family, and our study confirmed these results. Our study also demonstrated the in vitro bactericidal activity against *S. maltophilia* strains. No in vitro susceptibility testing of colistin was carried out on the small numbers of the intrinsically resistant gram negative isolates, in this study like the *Proteus*, *Burkhardia* and *Serratia* species. According to previous studies, polymyxins demonstrated no activity against Gram-negative or Gram-positive cocci, Gram-positive bacilli, or anaerobes.¹⁷ In this study, results interpreted according to CLSI breakpoint recommendations.⁸ So in our study, we did not perform in vitro susceptibility of colistin against these organisms.

There are several ways to interpret breakpoints for colistin susceptibility.⁶ As there is a limited number of studies evaluating the value of colistin in nosocomial infections caused by Gram-negative bacteria, determining a breakpoint for increased clinical success was a challenge in this study.^{11,12,14,18} Moreover, interpretation of resistance is further complicated by susceptibility criteria, which may vary from country to country.¹⁹ Data from 40 years ago established that colistin diffuses poorly in agar, resulting in highly unreliable DD.⁶ Our results showed that the incidence of very major errors of DD testing as compared to the Etest ranged between

1.4% in *A. baumannii* to 2.3% in *P. aeruginosa*, and the incidence of minor errors ranged between 0.7% in *A. baumannii*, to 8.3% in *S. maltophilia*, and 11.6% in *P. aeruginosa*. A recent study using 3 DD comparative methods indicated that 5-11% of results were categorized as very major errors, and the highest error (89%) was associated with *Enterobacter spp.*¹⁷ The variability in the rate of very major errors from one study to another related to the different methodology and breakpoints used to interpret the results. The rate of very major errors in DD testing can be reduced to 3.5-6% if the results are interpreted according to the criteria established by Gales et al (resistant ≤ 10 mm; susceptible ≥ 14 mm).¹⁵ However, when using these criteria, 71% of isolates fell into an intermediate susceptible category by DD and this findings was confirmed by the present study. These results emphasize the need for alternatives to the DD method in clinical laboratories. The Etest might present an attractive alternative method to DD, while routine MIC testing by the microdilution method in a clinical laboratory is tedious and time-consuming. Although the Etest is a simple and accurate method for determining antibiotic susceptibility, it has not yet been verified as an appropriate method for many strains with acquired colistin resistance. A recent study showed that the Etest has a concordance of 98.2% with broth microdilution on 115 isolates of *A. baumannii* with a minimal very major error rate (1.7%).²⁰ Other studies also showed the accuracy of Etest as compared with the DD test for susceptibility testing of colistin.^{21,22} In one of these studies it was suggested that doubtful results by DD should be confirmed by Etest.²³ We would also suggest that Etest results should be confirmed by a dilution method if the MIC is 1-2 $\mu\text{g/ml}$, and if colistin is required to treat a serious infection caused by *A. baumannii*. The accuracy of the Etest appeared to be greater than the accuracy of other MIC methods for *Enterobacteriaceae*, and less for *P. aeruginosa*.²³ This finding was also reported in a recent study comparing the Etest with agar dilution methods, where 7 of 12 strains of colistin-resistant *P. aeruginosa* were misclassified as susceptible by the Etest due to a 2-fold dilution shift towards lower MICs (6 of the 7 isolate MICs were 4 $\mu\text{g/ml}$) and the lack of an 'intermediate' category in this study.²¹ This discrepancy related mainly to indistinct breakpoints (2-4 $\mu\text{g/ml}$) and frequent MICs near the breakpoints for *P. aeruginosa* and *S. maltophilia*. To resolve this issue, the manufacturer suggests the reading of the MIC by extrapolating colonies from above the zone of inhibition to the Etest strip when testing the non-fermenting bacteria.⁶

One of the limitations of our study is the lack of correlation of clinical response of patients to colistin with the laboratory results, as most of our patients were

in the ICU's and there are many factors such as clinical conditions other than infections, which might affect there clinical or bacteriological response. Future larger scale multicenter laboratory clinical studies should be planned to address this issue.

We conclude that DD is an unreliable method to test colistin susceptibility in a clinical laboratory for seriously infected patients. We therefore recommended the Etest, which is a simple, reliable, and attractive alternative to reference methods for the detection of resistance to colistin in Gram-negative bacilli. In the rare cases of seriously ill patients of MICs near the breakpoints (1-2 µg/ml), confirmation of the result maybe carried out by broth or agar microdilution methods.

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