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Comparison of Broth Microdilution and Colistin Disk Elution Methods for the Determination of Colistin Susceptibility in Multidrug Resistant *Pseudomonas aeruginosa* Isolates

Çok İlaca Dirençli *Pseudomonas aeruginosa* İzolatlarında Kolistin Duyarlılığının Belirlenmesinde Sıvı Mikrodilüsyon ve Kolistin Disk Elüsyon Yöntemlerinin Karşılaştırılması

Neslihan ARICI[1], Nilgün KANSAK[1], Rıza ADALETİ[1], Sebahat AKSARAY[2]

[1]*University of Health Sciences Turkey, Haydarpaşa Numune Training and Research Hospital, Clinic of Medical Microbiology, İstanbul, Turkey*
[2]*University of Health Sciences Turkey, Hamidiye Faculty of Medicine, Department of Medical Microbiology, İstanbul, Turkey*

Abstract

Introduction: Colistin is increasingly used as a last-choice antimicrobial in the treatment of infections caused by multidrug-resistant *Pseudomonas aeruginosa* isolates. Due to the recent increase in colistin resistance around the world, the need for reliable methods for susceptibility testing has become more important. In this study, it was aimed to determine the performance of the broth disc elution (BDE) test, which was developed as an alternative to the reference broth microdilution (BMD) method.

Materials and Methods: Fourty eight multidrug resistant *Pseudomonas aeruginosa* strains isolated from various clinical specimens were included in the study. The BMD method was performed according to the recommendations of the ISO-standard (20776-1), and the BDE test was done according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. The categorical agreement, major error, and very major error (VME) rates of the BDE test were determined by comparing with the BMD method.

Results: By using the reference BMD method, 47 of 48 isolates were found to be susceptible (98%), and one was found to be resistant (2%). According to BMD test, the minimum inhibitory concentration distribution of the isolates was between 0.25-64 mg/l. The BDE test also correctly detected 47 susceptible and 1 resistant isolates. When the BDE test was compared with the BMD, the rate of categorical agreement was found to be 100%. No major or VMEs were detected.

Conclusion: The BDE test performed very well in determining colistin susceptibility in multidrug resistant *P. aeruginosa* isolates. Therefore, we think that the BDE test can be used as a practical and reliable method in laboratories with limited resources where it is not possible to perform the reference BMD method.

Keywords: *Pseudomonas aeruginosa*, colistin, broth disc elution, broth microdilution

Öz

Giriş: Kolistin, çok ilaca dirençli *Pseudomonas aeruginosa* izolatlarının neden olduğu enfeksiyonların tedavisinde son seçenek antimikrobiyal olarak giderek daha fazla kullanılmaktadır. Kolistin direncinin son zamanlarda tüm dünyada artması sebebiyle duyarlılık testleri için güvenilir yöntemlere olan ihtiyaç daha da önem kazanmıştır. Bu çalışmada, referans sıvı mikrodilüsyon (SMD) yöntemine alternatif olarak geliştirilmiş olan sıvı disk elüsyon (SDE) testinin performansının belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Çeşitli klinik örneklerden izole edilen toplam 48 adet çok ilaca dirençli *Pseudomonas aeruginosa* suşu çalışmaya dahil edildi. Sıvı mikrodilüsyon yöntemi, ISO standardı (20776-1) tavsiyelerine göre, SDE testi ise Klinik ve Laboratuvar Standartları Enstitüsü (CLSI) tavsiyelerine göre

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Address for Correspondence/Yazışma Adresi: Neslihan ARICI MD, University of Health Sciences Turkey, Haydarpaşa Numune Training and Research Hospital, Clinic of Medical Microbiology, İstanbul, Turkey
Phone: +90 553 349 66 80 **E-mail:** drnesliarici@gmail.com **ORCID ID:** orcid.org/0000-0003-4788-0044
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gerçekleştirildi. Sıvı disk elüsyon testinin kategorik uyum (KU), çok büyük hata (ÇBH) ve büyük hata oranları referans SMD yöntemi ile karşılaştırılarak belirlendi.

Bulgular: Referans SMD yöntemi ile 48 izolatın 47'si duyarlı (%98), 1'i dirençli (%2) olarak saptandı. Sıvı mikrodilüsyon testine göre izolatların minimum inhibitör konsantrasyon dağılımı 0,25-64 mg/l arasında idi. Sıvı disk elüsyon testi de duyarlı 47 izolat ile, dirençli 1 izolatı doğru olarak saptadı. Sıvı disk elüsyon testi SMD ile karşılaştırıldığında KU oranı %100 olarak bulundu. Büyük hata ve ÇBH saptanmadı.

Sonuç: Sıvı disk elüsyon testi, çok ilaca dirençli *P. aeruginosa* izolatlarında kolistin duyarlılığının belirlenmesinde oldukça iyi bir performans göstermiştir. Bu nedenle referans SMD metodunu uygulamanın mümkün olmadığı sınırlı kaynaklara sahip laboratuvarlarda, SDE testinin pratik ve güvenilir bir yöntem olarak kullanılabileceğini düşünüyoruz.

Anahtar Kelimeler: *Pseudomonas aeruginosa*, kolistin, sıvı disk elüsyon, sıvı mikrodilüsyon

Introduction

Due to the worldwide spread of multidrug-resistant *Pseudomonas aeruginosa* isolates, colistin is increasingly used as a last-line antimicrobial in the treatment of these infections[1]. Increasing use of colistin leads to the emergence of acquired colistin resistance. The most common mechanisms mediating acquired colistin resistance are chromosomal mutations and interspecies transmission of plasmid-derived *mcr* (1-9) genes. With the recent increase in both chromosomal and plasmid-induced colistin resistance all over the world, the need for reliable methods for susceptibility testing has become more important[2]. However, some technical difficulties are encountered in determining colistin susceptibility. The decrease in growth inhibition due to poor diffusion of colistin in agar due to its large molecular size adversely affects the performance of disc diffusion and gradient tests[3]. On the other hand, the very major error (VME) rates in the results obtained with automated systems (Vitek-2, Phoenix, etc.), which are frequently used in routine microbiology laboratories, make these methods unreliable[2]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[4] and the Clinical and Laboratory Standards Institute (CLSI)][5] defined the broth microdilution (BMD) method as the only reference in the determination of colistin susceptibility. However, broth microdilution method is rarely applied in routine microbiology laboratories because it requires qualified labor and is not a practical method. To overcome this difficulty, colistin broth disc elution (BDE) test, one of the alternative methods developed by Simner et al.[6] by the CLSI sub-working group, is an easy, simple and reliable method. There are few studies conducted in our country regarding the effectiveness of the BDE method on *P. aeruginosa* strains. In this study, it was aimed to determine the performance of the BDE test by comparing it with the reference BMD method.

Materials and Methods

Bacterial Isolates

A total of 48 multidrug resistant *P. aeruginosa* isolates, which were isolated from various clinical samples in our laboratory and stored at -80 degrees between August 2019 and March 2022, were included in the study. Multidrug resistance was defined as resistance to at least one antibiotic within three or more antibiotic categories. Identification of the isolates was performed with MALDI-TOF MS (Vitek MS, Biomerieux, France) and antibiotic susceptibility with Vitek-2 automated system (Biomerieux, France).

Broth Microdilution Method

Susceptibility tests were performed with 18-24 hour fresh bacterial cultures stored at -80 °C and passaged into sheep blood agar medium. Colistin sulfate salt (Sigma-Aldrich C4461, USA) was used for BMD testing according to ISO standards (20776-1)[7]. Minimum inhibitory concentration (MIC) values were examined in the dilution range of 0.125-128 mg/l. A stock solution was prepared by dissolving 25.6 mg of colistin in 10 ml of sterile distilled water (dH_2O). 1 ml of the stock solution was diluted with 9 ml of sterile dH_2O, and further dilutions were prepared from this 256 mg/l solution by serial dilutions. 50 µl of the prepared dilutions were dispensed into wells 1-11. Antibiotic solution was not added to well 12 because it was a microorganism growth control well. A suspension of bacteria at 0.5 McFarland turbidity ($1.5\text{-}2x10^8$ colony-forming units; CFU/ml) was prepared from fresh cultures in sterile saline. A 1:100 dilution was made using cation-adjusted Mueller Hinton Broth (CAMHB) (Mueller Hinton II Broth Cation-Adjusted BD 212322) (25 µl bacterial suspension + 2475 µl CAMHB) prepared according to manufacturer's recommendations. After 50 µl of the suspension containing 10^6 CFU/ml bacteria was dispensed into each well (1-12) (final bacterial concentration in the wells $5x10^5$), BMD plates were incubated at 35 °C for 18-24 hours. At the end of the incubation, the lowest colistin concentration without growth was calculated as the MIC value. Tests were repeated once again with inconsistent results.

Colistin Broth Disk Elution Method

The BDE study was performed in line with the CLSI recommendations[5]. For each isolate, 10 ml of cation-adjusted Mueller Hinton broth (Mueller Hinton II Broth Cation-Adjusted BD 212322) was added to 4 sterile glass tubes. The 1st tube, which was the growth control tube, was left empty, 1 piece of colistin discs (Bioanalyse, Turkey) were placed in the 2nd tube, 2

in the 3rd tube and 4 in the 4th tube, and the tubes were kept at room temperature for 30 minutes after gentle vortexing. 50 µl of 0.5 McFarland bacterial suspension was added to each broth without antibiotics and containing 1-2 and 4 mg/l colistin. BMD plates were evaluated after 18-22 hours of incubation at 35 °C, and BDE tubes after 16-20 hours of incubation. The lowest colistin concentration at which there was no growth was calculated as MIC.

Quality Control

It was performed using colistin-susceptible ATCC 27853 *P. aeruginosa* and colistin-resistant NCTC 13846 (mcr-1 positive) *E. coli* strains.

Evaluation of Results

For both tests, according to EUCAST version 12.0[4] recommendations, isolates with MIC ≤4 mg/l were considered susceptible, and isolates with >4 mg/l were considered resistant (Figure 1). The categorical agreement, major error (ME) and VME rates of the BDE test were determined by comparing them with the reference BMD method according to ISO criteria.

Statistical Analysis

The performance of the test was evaluated using the following parameters.

Categorical agreement: Accurate detection of susceptible as susceptible and resistant as resistant (≥90%).

ME: Detection of the isolate as resistant which was found susceptible by the reference method (<3%).

VME: Detection of the isolate as susceptible which was found resistant by the reference method (<3%).

Results

By using BMD method, 47 of 48 isolates were found to be susceptible (98%) and one was found to be resistant (2%). According to the reference BDE test, the MIC distribution of the isolates was between 0.25-64 mg/l. When the BDE test was compared with the BMD, the categorical agreement was found to be 100%. No VME or MEs were detected (Table 1).

Discussion

Currently, studies are continuing to discover reliable methods that will effectively detect colistin resistance arising from both chromosomal mutations and plasmid-derived *mcr* genes[2]. Simner et al.[6] stated that with the BDE test they developed in 2019, many existing obstacles in determining colistin susceptibility were overcome and they offered a practical, accurate and reproducible alternative for laboratories of all

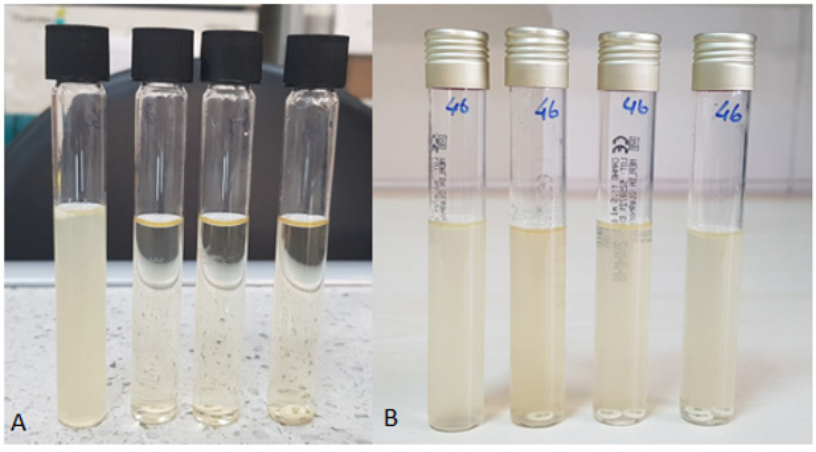

Figure 1. A) Colistin susceptible isolate (MIC ≤1) by using BDE test, B) Colistin resistant isolate (MIC >4) by using BDE test
BDE: Broth disc elution, MIC: Minimum inhibitory concentration

Table 1. Colistin MIC values obtained by using BMD and BDE methods in multidrug resistant *P. aeruginosa* isolates (n=48)

	BDE colistin MIC (mg/l)				
BMD colistin MIC (mg/l)		≤1	2	4	>4
	≤1	20			
	2	19	3		
	4	1	3	1	
	>4				1

BMD: Broth microdilution, BDE: Broth disc elution, MIC: Minimum inhibitory concentration

sizes. In their study, the categorical and basic agreement of the BDE test was found to be 100% in 24 *P. aeruginosa* isolates tested along with other microorganisms; no VME or ME were detected. However, it was emphasized that MICs could be determined lower by BDE method, especially in isolates carrying mcr-1, and when a 2 mg/l MIC value was determined by this method, the result should be confirmed with reference BMD.

Although many studies evaluated the performance of the test in detecting colistin resistance in *Enterobacterales* since the BDE was recommended by the CLSI in 2019, there were few studies covering *P. aeruginosa* isolates. Humphries et al.[8] found the categorical agreement of the BDE method for *P. aeruginosa* as 99.3%, while they did not detect any VME, they found the ME rate to be 0.7%. Based on these data, they stated that the BDE method was suitable for detecting colistin susceptibility in *P. aeruginosa* isolates. In the same study, they concluded that the BDE test should not be used for *Acinetobacter baumanni* due to the high VME rates obtained in these isolates. Butt et al.[9] also found the categorical and basic agreement of the test for *P. aeruginosa* isolates as 93.7% and 100%, respectively, and they did not detect a VME or a ME. In the light of these data, they concluded that the BDE test was a simple, practical and inexpensive susceptibility test and could replace BMD in routine microbiology laboratories. In the same study, it was reported that this method would not be useful in *A. baumannii* isolates due to the high VME rates (30%).

On the other hand, Dalmolin et al.[10] stated in their study that the BDE method was not reliable for non-fermenter Gram-negative bacteria, including *P. aeruginosa*, due to low categorical agreement and high VME rates. However, the use of *A. baumannii* strains other than *P. aeruginosa*, which were not approved for the BDE test, and the very low total number of strains (n=8) might cause the poor performance of the test.

In order to overcome the problem of low MIC value detected in *mcr* positive isolates by BDE method, various tests based on the inhibition of *mcr* activity by the addition of EDTA was also developed. Fenwick et al.[11] showed that the method could be used with high sensitivity (100%) and specificity (94.3%) for *mcr* screening by comparing the decrease in MIC values obtained with EDTA added BDE with MICs without EDTA. Although colistin resistance in our country is mostly of chromosomal origin rather than *mcr* genes transferred by plasmid, it should be considered by laboratories that these isolates can be encountered, albeit rarely.

When the studies conducted in our country were examined, it was seen that there were very few publications in which *P. aeruginosa* isolates were evaluated with the BDE test, and the number of isolates in these studies was lower than our study. In the study of Sarıkaya et al.[12], when the BDE test results of 24 *P. aeruginosa* isolates were compared with the BMD, the categorical agreement of the test was found to be 100% similar to our study, and no ME or VME was detected. Tüzemen et al.[13] developed a modified BDE method using less material to detect colistin resistance and compared the performance of the test with the reference method. In this method, two colistin discs for *Pseudomonas* spp. were placed in Mueller Hinton broth with 5 ml of cation added and the colistin BDE tube with a MIC value of 4 µg/ml was studied. 25 µl of the bacterial suspension prepared with turbidity equivalent to 0.5 McFarland standard turbidity was added to each tube and after incubation for 16-20 hours at 35 °C, colistin was interpreted as resistant in case of turbidity in this tube and sensitive in the absence of turbidity. A total of 15 *P. aeruginosa* isolates were studied and the categorical agreement of the test was 100%. Due to the difficulties of the BMD method, it has been stated that this method can be used in *Pseudomonas* spp. isolates in microbiology laboratories with high workload, but it cannot be used because of the high ME and VME rates in *Acinetobacter* spp. isolates.

Study Limitations

The fact that the BDE method with EDTA could not be applied in susceptible isolates due to technical limitations and the resistance genes were not investigated by molecular methods could be counted among the limitations of our study.

Conclusion

In conclusion, in our study, the BDE test showed a very good performance in determining colistin susceptibility in multidrug resistant *P. aeruginosa* isolates. However, considering the limitation of the BDE test in *mcr* positive isolates, testing the susceptible isolates with the BDE method with EDTA will provide more reliable results. In addition, we think that the BDE test can replace BMD as a very practical and reliable method in routine work in laboratories with limited resources where it is not possible to to perform the reference method.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Turkey, Haydarpaşa Numune Training and Research Hospital Clinical Research Ethics Committee on 17.04.2023 with the decision number HNEAH-KAEK 2023/KK/68.

Informed Consent: Informed consent was not obtained because the bacterial isolates were studied.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: N.A., N.K., Concept: N.A., N.K., R.A., S.A., Design: N.A., N.K., R.A., S.A., Data Collection or

Processing: N.A., N.K., Analysis or Interpretation: N.A., N.K., R.A., S.A., Literature Search: N.A., N.K., Writing: N.A.

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