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Review of Colistin Susceptibility Testing with Current Data

Kolistin Duyarlılık Testlerinin Güncel Bilgiler Eşliğinde İrdelenmesi

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Abstract

Polymyxin E is the main drug used in the treatment of microorganisms with multidrug resistance. The positively charged nature of colistin creates difficulties in susceptibility testing. As a result of the studies, the gold standard method to determine colistin susceptibility was determined as the broth microdilution method. However, this method has not yet entered the laboratory routine in many centers in our country. Our aim in this review was to examine methods that could detect colistin susceptibility, which was very important in treatment, in the light of current data. Disk diffusion method is not recommended due to the limited diffusion of the colistin molecule into the agar. While there are studies suggesting that E-test can be a reliable and suitable alternative when compared to the reference method, there are also studies that detect a very major error rate of over 3%. Automated methods have not been able to achieve the desired categorical agreement and very major error rates in many studies. Molecular-based methods are especially used to detect the *mcr* gene and are especially important in determining resistance transmission. The findings of newly developed rapid tests and methods such as colistin broth disk elution are encouraging, but more studies are needed in this area.
Keywords: Colistin, broth microdilution, susceptibility

Öz

Polimiksin E, çoklu ilaç direnci olan mikroorganizmaların tedavisinde kullanılan temel ilaçtır. Kolistinin pozitif yüklü yapısı duyarlılık testlerinde zorluklar yaratmaktadır. Yapılan çalışmalar sonucunda kolistin duyarlılığını belirlemek için altın standart yöntem sıvı mikrodilüsyon yöntemi olarak belirlenmiştir. Ancak bu yöntem henüz ülkemizde birçok merkezde laboratuvar rutinine girememiştir. Bu derlemede amacımız; tedavide oldukça önemli olan kolistin duyarlılığını saptayabilen yöntemleri güncel veriler ışığında inceleyebilmektir. Kolistin molekülünün agara difüzyonunun kısıtlı olması nedeni ile disk difüzyon yöntemi önerilmemektedir. E-test'in referans yöntem ile karşılaştırıldığında güvenilir ve uygun bir alternatif olarak görülebileceğini düşündüren çalışmalar olduğu gibi çok büyük hata oranının %3'ün üzerinde saptayan çalışmalar da mevcuttur. Otomatize yöntemler birçok çalışmada istenilen kategorik uyum ve çok büyük hata oranlarını yakalayamamıştır. Moleküler temelli yöntemler özellikle *mcr* genini saptamak için kullanılırlar ve özellikle direnç aktarımını belirlemede oldukça önemlidir. Yeni geliştirilen hızlı testler ve kolistin sıvı disk elüsyon gibi yöntemlerin bulguları yüz güldürücüdür, ancak bu alanda daha fazla çalışmaya ihtiyaç vardır.
Anahtar Kelimeler: Kolistin, sıvı mikrodilüsyon, duyarlılık

Introduction

The frequency of infections caused by multi-resistant Gram-negative bacteria is increasing worldwide[1]. Polymyxins, discovered in 1947, are among the oldest known antibiotics, initially limited in use due to their nephrotoxicity. However, they began to be widely used in the late 1960s due to the increasing number of multidrug-resistant strains (MDR) in the world. In recent years, microorganisms such as *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, which are susceptible only to polymyxins, have become a frequently encountered problem, especially in intensive care units, therefore the importance of polymyxins in parenteral form has increased[2]. Polymyxins are polypeptide antibiotics containing

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5 chemically different compounds (polymyxin A-E). Only two parenteral commercial forms, polymyxin E (colistin, PME) and polymyxin B, are used in clinical practice. PME is the commonly used type of polymyxin. PME is effective against many members of the *Enterobacterales* family, *A. baumannii* and *P. aeruginosa*. It is ineffective against Proteus, Gram-positive bacteria, gram-negative cocci and most anaerobes[2].

The lack of alternative drugs that can be used against MDR microorganisms is a serious problem for global world health. Unnecessarily used broad-spectrum antibiotics are known to be the most important factor in the development of resistance. Studies have shown that polymyxin, used in animals consumed as food, plays a major role in the spread of resistance. For this reason, many developed countries have banned the use of colistin in animal foods[3].

The positively charged structure of PME creates some difficulties in susceptibility tests [disk diffusion (DD), automated systems, etc.] that are frequently used in daily laboratory use. For this reason, the reference method recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) to determine colistin susceptibility is the broth microdilution test[4]. Our aim in this review was to examine antibiotic susceptibility methods that could detect PME resistance, which had a very important place in terms of world health, in the light of current data.

Structure and Mechanism of Action of Polymyxin E

Polymyxins are cyclic polycationic peptides. PME, which has amphipathic and cationic properties, is produced from *Bacillus colistinus*. PME contains the cyclic heptapeptide ring formed by D and L- amino acids together with the tripeptide side chain. It is covalently bonded to the fatty acid with the side chain acyl group[4].

Sodium colistin methanesulfonate (CMS) is obtained from colistin by the reaction of free γ-amino groups of diaminobutyric acid (Dab) residues with formaldehyde followed by sodium bisulfite and is the inactive prodrug of colistin[5]. In order for CMS to be activated, it must be hydrolyzed. Hydrolysis occurs rapidly at body temperature. The CMS form is suitable for intravenous, intramuscular, intrathecal and inhalation routes. Colistin sulfate form is only suitable for topical application[6].

The L-Dab, found in the structure of the strongly positively charged PME, binds to the negatively charged phosphate groups of lipid A, an important component of the lipopolysaccharide (LPS) of Gram-negative bacilli, through electrostatic interaction (since Gram-positive bacteria do not have LPS, they are naturally resistant to colistin). This interaction causes a competitive displacement of divalent cations of calcium and magnesium, resulting in destabilization of the inner cytoplasmic membrane. The resulting imbalance causes deterioration and damage to the structure of the outer membrane. As a result, due to membrane damage, the inside of the cell and the cytoplasm go outside and bactericidal activity occurs[7]. Deterioration in the bacterial membrane structure and increased permeability also increases the effect of other hydrophilic antibiotics (gentamicin, meropenem, tigecycline, etc.), and this mechanism is shown to be the reason for the synergistic effect[8].

Polymyxin E Resistance

Resistance to PME is commonly caused by modification of the LPS structure. The reason for the resistance developing with LPS modification may be some mutations in chromosomal genes or it may be related to plasmid-mediated encoded genes. Many genes and operons are involved in the modification of LPS. Most important ones; *pmrC* and *pmrE* genes, *PhoP/PhoPQ, PmrA/PmrB, mgrB* gene are plasmid-mediated mobile colistin resistance (*mcr*) genes[9]. For example, it has been determined that they contribute to resistance by activating the pmrA/pmrB regulatory system and leading to the regulation of the pmrCAB and arnBCADTEF-pmrE operons, which enable the synthesis and transfer of phosphoethanolamine (PEtn) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) molecules to lipid A, respectively, and subsequently, by indirectly activating the pmrA/pmrB regulatory system through pmrD[10,11]. Plasmid-mediated genes were first reported in *E. coli* isolated from pork and meat in China in November 2015 (*mcr-1* gene). Mcr-1 enzyme is a phosphoethanolamine transferase and adds phosphoethanolamine onto lipid A, leading to polymyxin resistance by altering LPS. Shortly after the discovery of Mcr-1, other mcr homologues (mcr-2-10) were reported and it was shown that resistance genes could be transferred horizontally between isolates[12,13]. This situation has shown that it may cause the global spread of resistance and has increased global public health concerns[14].

Determination Methods of Polymyxin E Susceptibility

The strong positively charged structure of PME, its insufficient diffusion in agar, and the heteroresistance of many Gram-negative microorganisms are the main reasons for the difficulty in detecting susceptibility[15].

Colistin minimum inhibitory concentration (MIC) values determined by the CLSI and EUCAST are summarized in Table 1[16,17]. European Committee on Antimicrobial Susceptibility Testing published a warning text for colistin susceptibility testing methods in 2016. This text, which was revised in 2019, included the following warnings:

Table 1. Current colistin MIC values determined according to CLSI and EUCAST

Microorganism	Colistin MIC value		
	Susceptible	Moderately susceptible	Resistant
CLSI			
Acinetobacter	-	≤2	≥4
P. aeruginosa	-	≤2	≥4
Enterobacteriaceae	-	≤2	≥4
EUCAST			
Acinetobacter	≤2	-	>2
P. aeruginosa	≤4	-	>4
Enterobacteriaceae	≤2	-	>2

CLSI: Clinical and Laboratory Standards Institute, EUCAST: European Committee on Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration

a) The DD method cannot be used for colistin susceptibility testing. This test cannot clearly distinguish between susceptible and resistant isolates.

b) Currently available gradient tests may underestimate colistin MIC values.

c) Users of semi-automatic devices should implement strict quality control and check with the manufacturer to ensure that colistin susceptibility methods provide accurate results.

d) Quality control should be performed with both a sensitive QC strain (*E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853) and colistin-resistant *E. coli* NCTC 13846 (mcr-1 positive)[18].

The methods used to determine colistin susceptibility are summarized below;

1. Dilutional Methods

As a result of the studies, the gold standard method recommended for colistin susceptibility is broth microdilution[4]. The main purpose of dilutional methods is to determine the MIC level as a result of the 16-24 hour incubation period. There are three types of dilution methods:

Broth microdilution method (BMD): It is considered the reference method. Sterile 96-well microplates are used for this method. Colistin sulfate is made into stock solution in accordance with the manufacturers' recommendations. 0.5 McFarland suspension is prepared from all isolates. First, cation-adjusted Mueller Hinton Broth (CAMHB) agar and the prepared antibiotic stock solution are added to the wells. The resulting mixture is pipetted by serial dilution. Then, the solution containing microorganisms is added. The inoculated plates are left to incubate. The MIC is the lowest antibiotic concentration value that completely inhibits bacterial growth (the first well that does not appear cloudy). Positive and negative controls must be checked before reading. Various easy-to-use automated commercial panels based on the BMD method have been developed, Sensititre™ (Thermo Fisher Scientific, Cleveland, USA) panel, MIC COL (Diagnostics, Galanta, Slovakia), but the reference method is considered the manual method[19].

There are several reasons that limit the use of the BMD method. It is quite laborious to do it compared to methods such as DD. Manual preparation of the antibiotic stock solution may cause errors, it is difficult to adapt to the routine, and problems may occur when reading the manual MIC value. Particularly, the phenomenon of skipping wells, which is observed in high concentrations while there is inhibition in the first wells, is thought to indicate heteroresistance and is one of the main difficulties experienced during reading[20].

Broth macrodilution method: It is the same as BMD in principle, only the process takes place in larger test tubes in larger volumes. 1 ml of CAMHB is placed in the tubes, then 1 ml of the stock antibiotic solution is added to the first tube and a two-fold dilution is made. Finally, an equal volume of bacterial suspension is added to all tubes and left for incubation. There is also a colistin broth disk elution (CBDE) method in which a colistin disk is used as the antibiotic source. In this method, an increasing number of colistin disks (10 µg) are added to the tubes containing CAMHB medium. Since antibiotic stock solution is not prepared in this method, the application of the method is more practical[21].

Agar dilution (AD): It is a method based on the principle of BMD, but using solid agar, which is equivalent to broth dilution. Mueller Hinton agar (MHA) medium supplemented with colistin at certain concentrations is used in a single petri dish with fixed compartments. It is not routinely recommended because it requires more labor than E-test and DD methods and the number of studies conducted is insufficient[22].

2. Diffusion Methods

Disk diffusion: The colistin DD test is performed by using a 10 µg colistin disk on MHA in which a solution containing a

certain density of microorganisms is inoculated. At the end of the incubation period, evaluation is made according to the diameter of the inhibition zone around the examined disk. However, the result can be determined qualitatively, the MIC value cannot be determined[23]. Due to its structure, colistin enters into electrostatic interactions with the acid and sulfate groups of the agar and diffuses weakly into the agar. Studies have shown that DD has high error rates in determining colistin susceptibility. For this reason, it has been reported that there is no reliable method to determine susceptibility to colistin[24].

Gradient test: It is a quantitative method. Similar to the DD method, an E-test strip impregnated with antibiotics is placed on the transplanted MHA. This strip contains increasing concentrations of antibiotics. The point where the ellipse-shaped inhibition zone meets the strip at the end of the incubation period is determined as the MIC. It is a simple and sensitive method to determine antibiotic susceptibility[25].

3. Automated Systems

Vitek-2 (bioMérieux, France), Phoenix etc. systems are colorometric, automated methods that can detect the MIC value. Minimum inhibitory concentration is determined by using a gram-negative antibiotic susceptibility card and performing a study as recommended by the company[26]. Compared to the reference method, automated systems showed variable performance between studies in detecting susceptibility to colistin. An important problem in automated systems is that the optimal MIC levels are not clear. In particular, borderline MICs must be confirmed by the reference method, regardless of the bacterial type[27].

4. Rapid Tests

In recent years, rapid polymyxin NP tests (RPNP) have been produced that can determine colistin resistance, especially in members of the *Enterobacterales* family. In this test, the CAMHB solution is divided into RPNP containing colistin (created by adding colistin stock solution) and RPNP solution without colistin. Afterwards, a solution containing colistin is placed in 4 wells and a solution without it is placed in 4 wells on the 8-well microplate. After the bacterial solutions are added, the incubated microplate is read to determine the MIC according to the color change of the wells[28]. The rapid test that can determine colistin resistance in non-fermentative bacteria is the rapid polymyxin NP (R-RPNP) test based on resazurin. Its working principle is the same as RPNP[29].

5. Polymerase Chain Reaction

The main purpose is to detect the genes that cause colistin resistance in the microorganism using molecular methods. In terms of colistin resistance, the polymerase chain reaction (PCR) method is generally aimed at detecting *mcr* genes. In addition, the PCR method plays a very important role in understanding the mechanism of colistin resistance spread and in conducting surveillance studies on resistant isolates[30].

Discussion

Today, PME is the most important antibiotic in the treatment of MDR microorganisms. Therefore, accurate determination of PME susceptibility is vital[3]. Studies have shown that plaque invasion is not good due to the cationic properties of PME, therefore the gold standard susceptibility determination method is BMD[4]. However, due to the difficulty of application, it has not been included in the routine of many laboratories in our country. Our aim in this review was to examine susceptibility methods for PME in the light of current data in the literature.

When examining antibiogram susceptibility tests, comparison is made according to the reference method. Errors made by the test compared to the reference method are evaluated in three categories: Very major error, major error and minor error. 'Major errors' are errors that have high impact on patient management, that is, the test shows strains that are actually resistant to be susceptible. According to CLSI, the very major error rate should be below 3%. The majör error is that it portrays susceptible strains as resistant. According to CLSI, this rate should again be below 3%. The minor error is that the test shows intermediately susceptible strains as susceptible or resistant. In addition, when comparing susceptibility tests with the BMD method, the proportion of isolates grouped in the same susceptibility category is calculated as categorical agreement (CA) and essential agreement (EA) and is expected to be over 90%[31].

The DD method is a simple, easily applicable and inexpensive method used to determine susceptibility to many antibiotics. In a study conducted with 228 isolates including *A. baumannii, P. aeruginosa* and *Enterobacterales* members, the DD method was compared with the reference method, and very high rates of resistant strains were found to be susceptible by the DD method[32]. In another study conducted in terms of colistin susceptibility, a very major error rate of 3.5% was reported when the DD method was compared with the reference method[33]. In addition, a reliable correlation between inhibition zone diameters around the disk and MICs has not yet been determined. For this reason, CLSI does not specify any zone diameter breakpoints for polymyxins and does not recommend the use of DD as a colistin susceptibility test[34].

In a study conducted in 2000, susceptibility was determined in 281 MDR microorganisms by the AD, DD and E-test methods, and the most successful method compared to the reference method was determined to be AD, and it was emphasized that AD was a reliable and reproducible method in determining the MIC of colistin[35]. Afterwards, studies on AD gained

momentum. In a study examining 61 carbapenem-resistant isolates, including *K. pneumoniae* and *A. baumannii* isolates, AD showed colistin susceptibility rates similar to BMD (4.9% vs 3.3%) with acceptable CA[36]. In a multicenter study, 270 isolates containing *P. aeruginosa, K. pneumoniae* and *A. baumannii* were tested with AD with 1-µl and 10-µl inoculum, and AD-10 had very major error rates for microorganisms, respectively; 0%, 0.5% and 14.3% were detected, and the error rates of AD-1 were found to be much higher. In this study, it was determined that 10-AD was the appropriate method to determine the colistin MIC value for *Enterobacterales* and *P. aeruginosa*[37]. In addition, in a study conducted on 401 *P. aeruginosa* strains obtained from patients with cystic fibrosis, it was observed that the AD method gave higher MIC rates than the reference method, and in this study, it was emphasized that BMD was a much more reliable method instead of AD[38]. It was thought that the reasons for the high MIC values might be MHAs with different cation concentrations. In light of all this, EUCAST and CLSI do not recommend the use of the AD method in routine colistin susceptibility determination.

Gradient methods are routinely used methods in laboratories. In previous years, there are studies showing a good correlation between the E-test method and BMD[39,40]. On the contrary, there are studies showing that E-test has a higher error rate than expected compared to the reference method[41]. In a comprehensive study, 6 susceptibility methods were compared and the highest discrepancy rate was found to belong to the E test[42]. Again, in a study comparing BMD with gradient assays, gradient assays were observed to underestimate colistin MICs, resulting in a significant number of falsely susceptible results (9-18 of 75 total tests, compared to 1-3 for BMD products)[42]. It was thought that this problem might be due to poor diffusion of polymyxins into the agar. The MIC compatibility problem in the E-test method was also emphasized in the warning text published by EUCAST in 2019.

There are many studies on the effectiveness of automated systems in determining colistin resistance. In a study conducted with the *Enterobacterales* family, Vitek-2 and the reference method were compared and the EA rate was found to be 93.4%, while the CA rate remained below 90% and the very major error rate was found to be 36%[43]. Another recent study included 778 colistin-resistant strains and compared the automated system with BMD. The highest error percentage was found for *A. baumannii* (very major error rate was 27.8%)[44]. In a study conducted in our country, the CA rate was found to be 92% and the very major error rate was 0%, according to the reference method[45]. In the comparison study conducted by Tanrıverdi Çaycı et al.[46] according to the reference method, the CA rate was found to be 84.12% and the very major error rate was found to be 55.88%.

In a study by Girardello et al.[27], Vitek-2 showed 90.1% baseline concordance and 91.4% CA for *Enterobacterales* isolates. However, a very major error rate of 7.9% was detected, and more successful results were obtained when ≤0.5 or ≥16 µg/ml values were determined as the limit value for MIC for *K. pneumoniae* and *E. coli*. A recent study conducted in Africa found a CA of 89%, a EA of 56% and a very major error rate of 55% with Vitek-2, and concluded that Vitek-2 was not an alternative to BMD as a colistin susceptibility test[47]. Therefore, in the light of these data, it cannot be said that automated systems are a reliable method to determine colistin resistance alone. Especially the MIC values that cannot be determined clearly pose the biggest question mark.

Molecular methods are used to detect *mcr* genes that have been shown to have an impact on the global spread of colistin resistance. However, they are not used as routine antibiotic susceptibility tests due to reasons such as being expensive and not being able to determine the MIC value. Studies have emphasized that molecular methods have 100% sensitivity and specificity and that it is important that they provide results in a short time[48]. However, it is thought that the primers commonly used for the PCR method that detects *mcr* genes may give incorrect results and some *mcr* genes may be missed in the tested samples. In some studies, new PCR primers were identified and presented to the literature[49].

When we look at new developments in colistin susceptibility methods, rapid tests attract attention. In a study published in 2022, the RPNP test was recommended as the first screening of colistin susceptibility testing due to its rapid results (≤3 hours), high sensitivity and specificity[50]. In a study comparing six susceptibility methods, it was shown that only the RPNP test could detect heteroresistant resistances[42]. The CBDE test, which is among the new methods and was described for the first time in 2019, is performed by adding colistin disks to tubes prepared similar to broth dilution methods. This method, which is more easily applicable, has been described as having similar effectiveness to the reference method[51]. In a study conducted in our country, EA and CA were detected to be 90% higher in CBDE compared to BMD, and the very major error rate was also found to be quite low[52].

Conclusion

As a result, in the light of current data, the gold standard in determining colistin susceptibility is the BMD method. However, since the test is difficult to adapt to routine in all centers and MDR gram-negative bacteria are a global health problem, the search for a more easily applicable susceptibility test continues. It can be said that future studies will especially focus on RPNP tests, CBDE method and gene identification studies.

Ethics

Authorship Contributions

Concept: A.A.B., Design: Y.N., A.A.B., Data Collection or Processing: Y.N., Literature Search: Y.N., A.A.B., Writing: Y.N.

Conflict of Interest: No conflict of interest was declared by the authors.

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