Original Research Article

The detection of colistin resistance in carbapenem resistant 
*Klebsiella pneumoniae* isolates

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ABSTRACT

**Background:** Infections that develop with resistant *Klebsiella pneumoniae* have been associated with high morbidity and mortality. Problems in the treatment of these infections have required the use of new treatment options such as colistin in recent years. This study was carried out to contribute to routine applications by investigating the compatibility of methods commonly used in determining colistin susceptibility with the gold standard broth microdilution (BMD) method.

**Methods:** Colistin MIC values were determined by BMD, broth microdilution based commercial kit (Sensititre, ThermoFisher, USA), Vitek2 (bioMerieux, France) and gradient test (Bioanalyse, Turkey) in 128 carbapenem resistant *K. pneumoniae* strains isolated from various clinical samples sent from July 2018 to July 2019 to the microbiology laboratory.

**Results:** According to the BMD method, 62 (48.4%) of the isolates were susceptible and 66 (51.6%) were colistin resistant. Accepting BMD method as reference, very large error (to find a susceptible result in a reference test as susceptible) and large error (to detect a susceptible result in a reference test as resistant) ratios in broth microdilution based commercial kit, automated system and gradient test were found respectively as 0%, 15.1, 18.1 and 0, 6.4 and 9.6%. According to the ISO performance criteria, the commercial kit shows “acceptable performance” while the other two tests have a very large error rate of >10% and are considered as “unacceptable performance”.

**Conclusions:** It was concluded that the ready kit we tested in laboratories with heavy workload could be used to determine the colistin susceptibility and the result determined in automated systems should be confirmed.

**Keywords:** Antimicrobial susceptibility test, *Klebsiella pneumoniae*, Colistin

INTRODUCTION

Infections caused by enteric gram-negative bacteria are frequently encountered in hospitalized patients, and rapid initiation of appropriate antibiotic therapy is important for the control of these infections due to high mortality rates. *Klebsiella pneumoniae* is a bacteria classified in the family *Enterobacteriaceae*, which can cause community and nosocomial infections (such as urinary tract infection, pneumonia, meningitis, sepsis). Especially these nosocomial pathogens are frequently seen in patients in intensive care unit. Resistance due to mechanisms such as ESBL (expanded spectrum beta-lactamase) and AmpC is common in *K. pneumoniae* and carbapenems are recommended for the presence of these resistance mechanisms.\(^1\) Detection of carbapenem resistance also required the use of new treatment options. Colistin (polymyxin E) is a polypeptide structure antibiotic that was excluded from the use in the 1980s due to nephrotoxicity and neurotoxicity, and has recently been reused as the last option in the treatment of multidrug-resistant bacteria. It acts on Gram-negative bacteria by binding to the lipopolysaccharide layer and disrupting the outer membrane structure.\(^2\) The defined colistin resistance
limit value for Klebsiella spp. is >2 mg/L. Colistin resistance can occur as a result of chromosomal mutation or with plasmid mediated resistance genes that can be easily transferred horizontally. Liu et al detected the mcr-1 gene, the plasmid-mediated colistin resistance mechanism, for the first time in 2015. Then mcr-2-8 genes were identified in human and animal samples. The plasmid-mediated mcr gene is a concern all over the world, especially due to its rapid spread and interspecific transfer. The European committee on antimicrobial susceptibility testing (EUCAST) suggested using the broth microdilution method (BMD) as a reference method for confirmation, since suspicious results were found in the automated system and in the interpretation of the results detected by the gradient test in the automated system. This study was carried out to determine colistin resistance rates in carbapenem resistant K. pneumoniae isolates and to compare the results of various tests used for this purpose.

METHODS

A total of 128 carbapenem-resistant K. pneumoniae strains isolated from various clinical samples (blood, urine, sputum, wound swab, pleural fluid, tracheal aspirate) was included in the study between July 2018 and July 2019. BacT/ALERT (BectonDickson, Maryland, USA) system was used to evaluate blood culture samples. Other sample types that were accepted to laboratory in accordance with the procedure and reproduction detected blood culture samples were evaluated at the end of 18-24 hours incubation at 37°C by passaging 5% sheep blood agar and EMB (eosin methylene blue) agar medium. Conventional methods and Vitek2 (bioMerieux, France) automated identification system were used to identify microorganisms and to determine antibiotic susceptibility. The isolates identified as K. pneumoniae and found to be resistant to carbapenems were kept in the “skim-milk” medium at -20°C. After the study period was completed, the isolates were freshly cultured for 18-24 hours to determine the minimum inhibitory concentration (MIC) values. Colistin MICs were determined with BMD method, commercial broth microdilution (Sensititre, ThermoFisher, USA), Vitrek 2 (bioMerieux, France), and the gradient test (Bioanalyse, Turkey). The results were evaluated according to EUCAST (2018; V.8.1) standards and isolates with a MIC value of ≤2 was considered as susceptible; isolates with a MIC value of >2 µg/ml as resistant.

Broth microdilution method

Colistin (Sigma-Aldrich, USA) was suspended according to the formula of “weight (mg)=volume (ml)* concentration (µg/ml)/potency (µg/mg)” and a stock solution was prepared as 1280 µg/ml. The prepared stock solutions were stored in eppendorf tubes at -20°C in small volumes. Stock solution was melted and used on the same day. Serial dilutions (0.125-64 µg/mL) were made to each well using cationated Mueller-Hinton broth in sterile U-based microplates. Bacteria colonies were collected and 0.5 McFarland (5x10⁸ cfu/mL) suspensions were prepared in saline (SF), and 100 µL of antibiotic dilutions were added and incubated at 35°C for 18-24 hours. As a positive control, antibiotics-free wells and bacteria-free wells were used as negative controls. Each microplate was covered with sterile plate sealer during incubation to prevent drying. The lowest concentration of colistin without reproduction was recorded as MIC value.

Broth microdilution based commercial kit

For the broth microdilution test (Sensitiser, Thermo Fisher, USA), bacterial inoculation was performed in the microplate wells containing lyophilised colistin in the range of 0.12-28 µg/ml according to the procedure, and the wells with reproduction in the plates were evaluated at 35°C after 18-24 hours incubation. The first well without reproduction was determined as MIC.

Automated system

MIC values detected after treated of K. pneumoniae isolates according to the procedure using (bioMerieux, France) AST-N326 antibiotic card were recorded.

Gradient test

Colistin (0.016-256 µg) strip was used according to the manufacturer’s recommendations. For this, a test strip was placed on the Mueller Hinton agar medium (Becton Dickson, Maryland, USA) from the bacterial suspension prepared at a 0.5 McFarland turbidity, after the medium was allowed to dry for 3-5 minutes. Plates were incubated at 37°C for 18-24 hours. At the end of the incubation, the plates were evaluated by eye on a dark background in a bright environment, and the concentration at which the full inhibition zone touched the gradient test strip was recorded as the MIC value. The MIC values determined and the values of MIC50 (minimum inhibitory concentration value that inhibits 50% of the strains tested) and MIC90 (minimum inhibitory concentration value that inhibits 90% of the strains tested) were calculated using the Microsoft excel 2007 version.

Statistical analysis

Minor error, major error and very major error rates were determined for the verification of the tests (10).

Minor error

Determination of the result found as susceptible or resistant by the reference method to be moderately susceptible by the other antibiotic susceptibility test.

Major error

Determination of the result found as susceptible by the reference method to be resistant by the other antibiotic susceptibility test.
**Very major error**

Determination of the result found as resistant by the reference method to be susceptible by the other antibiotic susceptibility test.

BMD method was accepted as the gold standard for comparison. According to the EUCAST recommendation, the acceptable performance was accepted as <10% for very major error and major error according to the criteria determined by ISO in order to compare the verification between the methods. BMD results are accepted as reference and the compatibility of commercial kit, gradient test and automated system test results were evaluated.

**RESULTS**

A total of 128 carbapenem resistant *K. pneumoniae* strains isolated from various clinical samples were included in the study. The distribution of the isolates according to sample types was given in Table 1.

**Table 1: Distribution of *K. pneumoniae* strains by sample types.**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>42 (32.8)</td>
</tr>
<tr>
<td>Urine</td>
<td>35 (27.3)</td>
</tr>
<tr>
<td>Tracheal Aspirate</td>
<td>25 (19.5)</td>
</tr>
<tr>
<td>Wound Swab</td>
<td>13 (10.2)</td>
</tr>
<tr>
<td>Sputum</td>
<td>12 (9.4)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>Total</td>
<td>128 (100)</td>
</tr>
</tbody>
</table>

Comparison of BMD method and commercial kit, automated system and gradient results are given in Table 2.

**Table 2: Comparison of BMD method and other methods for Colistin susceptibility.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Susceptible N (%)</th>
<th>Resistant N (%)</th>
<th>Major error N (%)</th>
<th>Very major error N (%)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD</td>
<td>62 (48.4)</td>
<td>66 (51.6)</td>
<td>Reference method</td>
<td>Reference method</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>Vitek&lt;sup&gt;2™&lt;/sup&gt;</td>
<td>68 (53.1)</td>
<td>60 (46.9)</td>
<td>4 (6.4)</td>
<td>10 (15.1)</td>
<td>&lt;1</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Gradient test</td>
<td>68 (53.1)</td>
<td>60 (46.9)</td>
<td>6 (9.6)</td>
<td>12 (18.1)</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Commercial kit</td>
<td>62 (48.4)</td>
<td>66 (51.6)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>128</td>
</tr>
</tbody>
</table>

According to the BMD method, 62 (48.4%) of the isolates were susceptible and 66 (51.6%) were found to be colistin resistant. Considering the BMD method as a reference, the very major and major error rates were determined respectively as 0, 15.1, 18.1%, and 0, 6.4, 9.6%, in commercial kit, automated system and broth microdilution based gradient test. According to ISO performance criteria, the commercial kit shows "acceptable performance" while the other two tests have a very major error rate of 10% and are considered as "unacceptable performance".

**DISCUSSION**

The mortality rate of infections caused by Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), is high. Especially in the last decade, the increase in carbapenem resistance has reached remarkable dimensions. The carbapenem resistance for *E. coli* and *K. pneumoniae* in Turkey is respectively as 1-5 and 25-50% in central Asian and European surveillance of antimicrobial resistance (CAESAR) 2017 report prepared by world health organization. With the wide spread use of colistin against carbapenem-resistant *K. pneumoniae* infections, resistant antibodies to this antibiotic group were also started to be detected in a short period. European surveillance of antimicrobial consumption network (ESAC-NET) reported that the colistin resistance increased double-fold in carbapenem resistant *Enterobacteriaceae* family between 2009 and 2013. Sizülük et al evaluated colistin-phosphomycin resistance in carbapenem-resistant gram-negative bacteria in their study on 147 Klebsiella isolates, and found colistin resistance as 76.1%. Menekşe et al found the colistin resistance as 53.3% by broth microdilution method in carbapenem resistant Klebsiella isolates in their study to examine the relationship between colistin resistance and mortality. Capone et al determined the colistin resistance rate as 20.4% in *Klebsiella* strains (n: 97) in a multi-center study using broth microdilution method. Monaco et al found 76 out of 178 isolates (43%) resistant to colistin in their study covering 45 centers using broth microdilution method. In our study, colistin resistance was found to be 51.6%.

In a study conducted by Kaza et al, it was found that colistin resistance increases morbidity and 6 times more colistin resistance in *K. pneumoniae*. In their study, Güdükçüoğlu et al found that the 14-day survival rate in colistin resistant *K. pneumoniae* was 40%. In their study, Menekşe et al found that increased colistin MIC values in carbapenem resistant gram negative bacteria significantly decreased the 30-day survival rate (p=0.029). In their study, Rojas et al found that the mortality rate was 39% with colistin susceptible agents and 51% with colistin resistant bacteria in 30-day follow-up. In their 4.5-year follow-up study, Giacobbe et al found the mortality rate as 51% due to colistin use in 142 carbapenem resistant Enterobacteriaceae family between 2009 and 2013.
Klebsiella isolate. Due to the correlation between mortality and colistin susceptibility in vitro, EUCAST recommends using broth microdilution as a susceptibility test for colistin. Disc diffusion, gradient test and automated systems also give false results due to the structure of the colistin, and do not distinguish resistant and susceptible ones. Accepting the BMD method as the reference method of the 128 isolates (51.6%) was found colistin resistant in our study. According to the ISO performance criteria, the commercial kit shows "acceptable performance" while the other two tests are considered as "unacceptable performance" due to having a very major error rate of >10%. Accordingly, it was found that the results determined to be susceptible in both tests should be confirmed, and that the results found to be resistant could be reported due to the acceptable limit. Broth microdilution method requires attention and experience during application and interpretation. The ease of use of ready-made commercial kits in laboratories with high workload is an advantage. New generation liquid microdilution-based tests are faster and easier to apply than reference broth microdilution. In our study, the susceptibility of the ready broth microdilution-based test was found 100%.

Since automated systems and gradient tests can be performed in clinical laboratories without requiring any extra equipment and experience, they are an alternative to reference BMD method in terms of time and ease of use of broth microdilution based commercial kits.

We think that working with more isolates of other species in the Enterobacteriaceae family will contribute to the literature. In this study, it has been shown that since the error rates in these tests are very high for the colistin susceptibility, colistin susceptibility should not be reported except for those obtained with broth microdilution in order not to mislead patient treatment.

CONCLUSION

It was concluded that the ready-made kit that we tested was available in the laboratories with heavy workload to determine the colistin susceptibility, and the result determined in automated systems should be confirmed.

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Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES
