ORIGINAL ARTICLE

Evaluation of Colistin and Tigecycline Susceptibility Testing Methods for Klebsiella pneumoniae and Acinetobacter baumannii Clinical Isolates

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Key words: Colistin, Tigecycline, Klebsiella pneumoniae, Acinetobacter baumannii

ABSTRACT

Background: The rise of nosocomial infections caused by multidrug-resistant (MDR) and extended drug-resistant (XDR) Acinetobacter baumannii and Klebsiella pneumoniae are seen as a severe public health problem due to the restricted treatment choices. Colistin and tigecycline are increasingly used as a last choice for treatment of these infections. The most accurate antibiotic susceptibility methods for colistin and tigecycline are still challenging. Objectives: to detect colistin and tigecycline antibiotic susceptibility of K. pneumoniae and A. baumanii and evaluate disk diffusion (DD), E-test and VITEK 2 automated system compared to broth dilution (BD) test. Methodology: This study was performed on 35 K. pneumoniae and 15 A. baumanii clinical isolates collected from patients admitted to Benha University Hospitals. The isolated strains were identified by the standard laboratory technique with subspecies identification by VITEK 2 automated system. Colistin and tigecycline antibiotic susceptibility for K. pneumoniae and A. baumanii were evaluated by E-test, disk diffusion and VITEK 2 compared to BD as the reference method. Results: Through the study of the studied k. pneumoniae and A. baumanii strains, The essential and categorical agreements of colistin susceptibility were (82% & 80 %) for E-test, (92% & 98%) for VITEK 2 and categorical agreement for DD was 54%. The essential and categorical agreements of tigecycline susceptibility were (96% & 98%) for E-test, (88% &78%) for VITEK 2 and categorical agreement for DD was 74%. Conclusion: For colistin, VITEK 2 is considered a reliable method to detect colistin susceptibility while E-test and disk diffusion showed a poor performance. For tigecycline, E-test showed the best performance compared to the gold standard test while shortcomings of automated VITEK 2 and manual DD were observed.

INTRODUCTION

Klebsiella pneumoniae (k. pneumoniae) and Acinetobacter baumannii (A. baumannii) are a great cause of hospital acquired infections (HACIs), and is especially prevalent in intensive care units (ICUs). They are a frequent cause of hospital-acquired pneumonia and ventilator-associated pneumonia. The usage of antibiotics can be a factor that increases the HACIs with these organisms 1,2.

Formerly, it was thought that carbapenems were the most effective against multidrug-resistant (MDR) gram-negative bacteria, and they were used often due to their low toxicity and great efficacy 3. However, the excessive usage of carbapenems during the last ten years has led to a fast development of carbapenem-resistant bacteria. Since the majority of these strains are also resistant to at least one agent in the majority of other broad-spectrum antibiotic classes, they are classified as extended drug-resistant (XDR) 4,5.

The present study aimed to detect colistin and tigecycline antibiotic susceptibility of K. pneumoniae and A. baumanii and evaluate disk diffusion (DD), E-test and VITEK 2 methods compared to broth dilution (BD) method.

The increasing occurrence of MDR and XDR A. baumannii and Enterobacteriaceae infections led to the re-use of ancient antibiotics that may be still active against them, such as colistin 6.

Colistin is increasingly used as a last-resort treatment for infections caused by MDR and XDR pathogens, particularly carbapenem-resistant (CR) gram-negative bacteria 7. In recent years, however, colistin resistance has increased extensively, notably in K. pneumoniae and A. baumannii baumannii clinical isolates 5. Colistin resistance has been linked to the deletion or modification of the lipopolysaccharide (LPS) molecule due to mutations in the pmrCAB operon 8,9,10,11.

Tigecycline is the first member of the antibacterial glycyclycline family. It is a derivative of minocycline. By binding reversibly to the 30S subunit of the bacterial ribosome, this drug suppresses protein translation and amino acid synthesis 12.

Tigecycline is also considered one of the last choices to treat MDR and XDR bacterial infections. The increased use of it led to emerging of its resistance rapidly 13.
METHODOLOGY

This trial was performed in the Medical Microbiology and Immunology Department, Faculty of Medicine, Benha University over a period of one year from December 2019 to November 2020.

The current study was done on 35 strains of *K. pneumoniae* and 15 A. baumanii. The clinical samples included: (15) broncho-alveolar lavage, (18) sputum, (15) urine, (2) lung aspirate. The samples were collected from ICU and Chest Departments of Benha University Hospitals. The patients were 20 females and 30 male patients, their ages ranged from 20-80 years old.

Written consent was obtained from all participants involved in the study after the protocol was approved by the institutional ethical committee.

Isolation and identification of *Klebsiella* and *Acinetobacter* species

Clinical samples were cultured on MacConkey’s and CLED agar plates and incubated at 37°C for 24 hours. The growing organisms were identified as *Klebsiella* and *Acinetobacter* by the standard laboratory technique including: colony morphology, gram staining, sugar fermentation tests and oxidase reaction.

Identification of *K. pneumoniae* Subspecies pneumoniae and *A. baumanii* were done using VITEK® 2 Systems identification cards (BioMerieux, France).

Antibiotic susceptibility and antibiogram:

**Antibiotic susceptibility by disk diffusion (DD):**

Disk diffusion test was done for all isolates by using a sterile swab, the bacterial colonies were inoculated on the plates of Mueller Hinton agar after dipping the swab in the bacterial suspension adjusted to 0.5 McFarland.

Using sterile forceps, the antibiotic discs were placed in the center of the Mueller Hinton agar plates and gently pressed to create optimum contact. The agar plates were inoculated aerobically at 35°C for 16-18h. The discs were Ampicillin/sulbactam (SAM20 = 10/10µg), Tetracycline (TE = 30µg), Ceftriaxone (CRO=30µg), Imipenem (IPM=10µg), Colistin Sulphate (CS=10µg), Tigecycline (TGC=30µg).

**Antibiotic susceptibility by Broth dilution (BD) test:**

This procedure involved preparing twelve tubes. The first eleven tubes was prepared by two-fold dilutions of antibiotics (from 128 to 0.125 µg/mL) in a standard broth medium. The antibiotic-containing tubes were inoculated with a standardized bacterial suspension equivalent to 0.5 McFarland standard except the tube number eleven (used as negative control for turbidity). The tube number twelve was prepared by only a bacterial suspension broth equivalent to 0.5 McFarland standard without antibiotics used as a positive control tube for turbidity. Following overnight incubation at 35°C±2 C, the tubes were checked for turbidity. The antibiotic powders used were colistin sulphate and tigecycline.

**Antibiotic susceptibility by E-test strips:**

E-test method was done for all isolates by using a sterile swab, the bacterial colonies were inoculated on the plates of Mueller Hinton agar after dipping the swab in the bacterial suspension equivalent to 0.5 McFarland.

E-test strips were applied to the agar surface using sterile forceps. The strip was placed with the ‘E end’ facing upwards. The strips were colistin (0.016-256) µg/mL and tigecycline (0.016-256) µg/mL (BioMerieux, France). Plates were incubated aerobically at 37°C for 18-24 hrs (figure 2-3).

**Antibiotic susceptibility by VITEK 2 system:**

**Fig. 1:** Antibiotic susceptibility by disk diffusion method.

**Fig. 2:** Colistin E-test

**Fig. 3:** Tigecycline E-test
The antibiotic susceptibility testing (AST) for the VITEK-2 System is an automated test based on the MIC approach described by MacLowry and Marsh and Gerll. Before rehydrating the antimicrobial medium inside the card, the organism suspension is diluted to a standardized concentration in 0.45 percent saline. After stacking and sealing the card, it was inserted into the instrument incubator/reader VITEK-2 system. For 18 hours, the system measured the development of microbes in each well of the card. At the conclusion of the incubation period, the minimum inhibitory concentrations (MICs) of each antibiotic on the card were calculated. This card was an AST-XN05 (BioMerieux, France).

Interpretation of results and data analysis: the CLSI provides susceptibility breakpoints for colistin (susceptible, MIC of < 4 µg/mL; resistant, MIC of ≥ 4 µg/mL and zone diameter of susceptible ≥ 11 mm; resistant ≤ 10 mm) but don't provide breakpoints for tigecycline. FDA breakpoints for tigecycline (susceptible, MIC of ≤ 2 µg/mL; intermediate, MIC of ≥ 4 µg/mL; resistant, MIC of ≥ 8 µg/mL and zone diameter of susceptible ≥ 19 mm; intermediate 15-18 mm; resistant ≤ 14 mm).

The outputs of the DD, E-test, and VITEK 2 procedures for colistin and tigecycline were compared to the gold standard to evaluate the data. EA for BD was defined as the fraction of MICs within 1 dilution of doubling MIC for BD. Categorical agreement (CA) is the proportion of isolates allocated by both BD and the assessed method to the same susceptibility category. Minor errors (MinEs) identified susceptible isolates vs intermediate isolates and intermediate isolates versus resistant isolates. The International Organization for Standardization established acceptable performance according to the following criteria: more than ninety percent for essential or category agreement, fewer than three percent for VMEs or MEs, and less than seven percent for MEs plus MinEs.

RESULTS

Susceptibility to colistin, by BD, 22 % of isolates were colistin resistant, 20% among K.pneumoniae and 26.7% among A. baumannii. Discordant susceptibility rates (38% resistance rate) for E-test with interpretative errors and unacceptable EA and CA were observed (82 & 80%) with high ME (18%). VITEK 2 categorized 24% of isolates as colistin resistant showing excellent overall EA and CA compared to BD (92 & 98 %), low MEs (2%) with no VMEs. DD generated low CA (54%) with high MEs (46%). The data are shown in Table-1.

Table 1: Colistin susceptibilities of the studied isolates by susceptibility methods and EA, CA, and types of errors produced by E-test, VITEK 2, and DD compared with BD method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Susceptible NO(%)</th>
<th>Resistant NO(%)</th>
<th>EA NO(%)</th>
<th>CA NO(%)</th>
<th>VMEs NO(%)</th>
<th>MEs NO(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All isolate s (NO =50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>39(78)</td>
<td>11(22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-test</td>
<td>31(62)</td>
<td>19(38)</td>
<td>41(82)</td>
<td>40(80)</td>
<td>1(2)</td>
<td>9(18)</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>38(76)</td>
<td>12(24)</td>
<td>46(92)</td>
<td>49(98)</td>
<td>0(0)</td>
<td>1(2)</td>
</tr>
<tr>
<td>DD</td>
<td>16(32)</td>
<td>34(68)</td>
<td></td>
<td>27(54)</td>
<td>0(0)</td>
<td>23(46)</td>
</tr>
<tr>
<td>K. pneumoniae (NO= 35)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>28(80)</td>
<td>7(20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-test</td>
<td>21(60)</td>
<td>14(40)</td>
<td>31(88.6)</td>
<td>28(80)</td>
<td>0(0)</td>
<td>7(20)</td>
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<tr>
<td>VITEK 2</td>
<td>27(77.1)</td>
<td>8(22.9)</td>
<td>32(91.4)</td>
<td>34(97.2)</td>
<td>0(0)</td>
<td>1(2.8)</td>
</tr>
<tr>
<td>DD</td>
<td>12(34.3)</td>
<td>23(65.7)</td>
<td></td>
<td>19(54.3)</td>
<td>0(0)</td>
<td>16(45.7)</td>
</tr>
<tr>
<td>A.baumannii (NO = 15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>11(73.3)</td>
<td>4(26.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-test</td>
<td>10(66.7)</td>
<td>5(33.3)</td>
<td>10(66.7)</td>
<td>12(80)</td>
<td>1(6.7)</td>
<td>2(13.3)</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>11(73.3)</td>
<td>4(26.7)</td>
<td>14(93.3)</td>
<td>15(100)</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>DD</td>
<td>4(26.7)</td>
<td>11(73.3)</td>
<td></td>
<td>8(53.3)</td>
<td>0(0)</td>
<td>7(46.7)</td>
</tr>
</tbody>
</table>

*NO: number of the strains.

Susceptibility to tigecycline: by BD, 12 % of overall isolates were tigecycline resistant, 11.1 % among K. pneumoniae and 13.3 % among A. baumannii. EA and CA were high for E-test (96 & 98% overall) with low MinEs (2%), with no MEs and VMEs, exceeding the 8%). The data are shown in Table-2.
**Table 2:** Tigecycline susceptibilities of the studied isolates by susceptibility methods and EA, CA, and types of errors produced by E-test, VITEK 2, and DD compared with BD method.

<table>
<thead>
<tr>
<th>Method</th>
<th>All isolates (NO=50)</th>
<th>K. pneumoniae (NO= 35)</th>
<th>A.baumannii (NO = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible NO(%)</td>
<td>Intermediate NO(%)</td>
<td>Resistant NO(%)</td>
</tr>
<tr>
<td>BD</td>
<td>40(80)</td>
<td>4(8)</td>
<td>6(12)</td>
</tr>
<tr>
<td>E-test</td>
<td>40(80)</td>
<td>3(6)</td>
<td>7(14)</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>34(68)</td>
<td>5(10)</td>
<td>11(22)</td>
</tr>
<tr>
<td>DD</td>
<td>29(58)</td>
<td>8(16)</td>
<td>13(26)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>BD (28(80)</td>
<td>3(8.6)</td>
<td>4(11.1)</td>
</tr>
<tr>
<td></td>
<td>28(80)</td>
<td>3(8.6)</td>
<td>4(11.1)</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>25(71.4)</td>
<td>4(11.4)</td>
<td>6(17.1)</td>
</tr>
<tr>
<td>DD</td>
<td>20(57.1)</td>
<td>6(17.1)</td>
<td>9(25.7)</td>
</tr>
<tr>
<td>A.baumannii</td>
<td>BD (12(80)</td>
<td>1(6.7)</td>
<td>2(13.3)</td>
</tr>
<tr>
<td></td>
<td>12(80)</td>
<td>0</td>
<td>3(20)</td>
</tr>
<tr>
<td>VITEK 2</td>
<td>9(60)</td>
<td>1(6.7)</td>
<td>5(33.3)</td>
</tr>
<tr>
<td>DD</td>
<td>9(60)</td>
<td>2(13.3)</td>
<td>4(26.7)</td>
</tr>
</tbody>
</table>

*NO: number of the strains.

**DISCUSSION**

The rising incidence of multidrug-resistant and extensively drug-resistant A. baumannii and K. pneumoniae has boosted the use of colistin and tigecycline as a last-resort therapy for infections caused by these organisms, especially carbapenem-resistant gram-negative bacteria. In recent years, however, colistin and tigecycline resistance has dramatically grown, particularly among K. pneumoniae and A. baumannii.

It is difficult to choose an AST method for colistin because of its poor penetration into agar media. CLSI and FDA have promoted BD as the preferred technique for MIC determination, despite the fact that it needs trained personnel with advanced pipetting skills and precise digital weighing equipment, which some clinical microbiology labs may lack. To provide appropriate treatment recommendations, clinical labs must conduct accurate and timely colistin susceptibility tests. Few research have examined colistin susceptibility tactics to date, yielding equivocal findings; hence, defining the ideal approach remains challenging.

The VITEK2 system is fully automated and detects species identification and antibiotic susceptibility testing for several clinical isolates. It has lately been adopted by other clinical microbiology laboratories throughout the world. Laboratories that do not have automated AST methods, often use E-test and DD test for colistin AST. Commercial BD methods are less used in laboratories and have largely been found to be reliable by many laboratories as well.

Also, tigecycline AST is of major importance for the appropriate outcomes. The decreased treatment choices for infections by MDR and XDR bacteria ensure the accurate tigecycline susceptibility methods importance. The usage of AST has been concerned in controversies due to the reporting of MEs and more specifically, VMEs.

The present study reported a high resistance rate of *K. pneumoniae* and *A. baumannii* to different antibiotics i.e. Piperacillin, Ticarcillin/Clavulnic Acid, Cefuroxime, Cefixime, Ceftriaxone, Cefepime, Aztreonam while lower resistance rate were detected to Chloramphenicol, Minocycline, Meropenem, Levofloxacin, Trimethoprim and the lowest resistance rates were with tetracycline, tigecycline and colistin respectively.

In the current study, E-test exhibited a poor performance in colistin resistance when compared to BD as a gold standard test among total isolates with EA, CA, MEs and VMEs (82%, 80%, 18% and 2%) respectively. For *K. pneumoniae*, low EA, CA (88% & 80%) and high MEs 20 %. For *A. baumannii*, low EA, CA (66.6% & 80%) high MEs and VMEs (13.3% & 6.7%) respectively. Dafopoulou et al., Bakthavatchalam et al. and Hindler and Humphries supported our E-test limitations among total isolates. Chew et al. and Leilouche et al. on *Enterobacteirae* and *A. baumannii* reported low EA and high MEs and VMEs. It is probable that the poor performance of the E-test is attributable to the poor diffusion of polymyxin molecules, which results in a restricted inhibitory zone.

VITEK 2 exhibited excellent performance among overall total isolates i.e. high CA and EA (98% & 94%) and low MEs (2%) and no VMEs. Dafopoulou et al. reported that VITEK 2 exhibited appropriate performance for colistin susceptibility among *K. pneumoniae* and *A. baumannii*. Lee et al., Dafopoulou et al. and Singhal et al reported that the CA of the VITEK 2 test was 100%, 90 and 100% respectively with no MEs for *A. baumannii*.

On other hand studies done by Chew et al. and Leilouche et al. on *Enterobacteirae* and *A. baumannii* reported a poor performance of VITEK 2 method due to high VMEs.

DD exhibited a poor performance for detection of colistin susceptibility among total isolates i.e. CA and ME (54% & 46%). Also several studies reported a high rate of very major errors of DD test for colistin susceptibility varied from 5 to 11%.
In the current study, when E-test was compared with BD test for detection of tigecycline susceptibility, it exhibited appropriate performance for total isolates with EA, CA, MEs, VMEs, MinEs (96, 98, 0, 0 & 2%) respectively. For K. pneumoniae, the CA and EA were 100% & 94.3% with no MEs, VMEs and MinEs at all. For A. baumanii, the CA and EA were high 93.3 and 100% and low MEs, VMEs and MinEs (0 , 0& 6.7%) respectively. Lat et al. 27 study stated that E-test is reliable for tigecycline susceptibility among A. baumanii with high MIC agreement 94%. Zarkotou et al. 28 and Zhang et al. 29 reported appropriate performance of E-test for detection of tigecycline resistance among K.pneumoniae with high EA and CA. however Bedenić et al. 30 reported low level of CA and EA > 90% with high MEs and MinEs.

In this study, VITEK 2 exhibited a low performance among total studied isolates i.e. low CA and EA (78% & 85%) and high MEs and MinEs (4 & 16%) among K. pneumoniae i.e. low CA, EA was 80 %, 88.6 % and high MinEs 17.1%. and among A. baumanii i.e. CA, EA, MEs and MinEs 73.3%, 86.6%,13.3% & 13.3% respectively. Zarkotou et al. 28, Lat et al. 31, Zhang et al. 32 and Idleveich et al. 33 agreed with this study as they reported a poor performance of VITEK 2 to detect tigecycline susceptibility among K. pneumoniae with higher resistance rate than BD method. Şimşek and Demir 33 reported a similar poor performance of VITEK 2 for tigecycline susceptibility among A. Baumanii.

DD showed poor performance among total isolates i.e. low CA (74%), ME, and MinEs (8 & 18%) respectively. For K. pneumoniae, CA, MEs, VMEs and MinEs were 71.4, 8.5, 0, 20% respectively. For A. baumanii, the CA, MEs, MinEs was 80, 6.7 and 13.3% respectively. Zhang et al. 34 also reported poor performance of DD. Nageeb et al. 34 reported DD test limitation to detect tigecycline susceptibility are determined on Mueller-Hinton agar which contains manganese at concentrations higher than 8 mg/L which may produce falsely elevated resistance rate.

CONCLUSION

This study highlights the crucial role of antibiotic susceptibility methods for colistin and tigecycline. Important limitations of the E-test gradient diffusion and disk diffusion tests, which may result in inappropriate selection of colistin treatment, were perhaps the most significant finding of this research. Therefore, labs must exercise caution with these findings and do the BD test for colistin susceptibility, particularly when colistin treatment is necessary. When the BD test is difficult to execute, colistin susceptibility is best determined using automated equipment such as VITEK 2.

The E-test susceptibility test outperformed the gold standard BD test for Tigecycline. The current research demonstrates the poor performance of the VITEK 2 and DD susceptibility tests, which may considerably reduce available treatment options or lead to erroneous medication, and thus must be validated by the BD test, especially when tigecycline therapy is required.

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