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ORIGINAL ARTICLE

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

Roshdan M. Arafa, Abeer A.Aboelazm, Mona M. Saleh*, Hasnaa S. Abd Elhameed
Department of Medical Microbiology and Immunology, Faculty of Medicine, Benha University

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****Corresponding Author:***
Mona Mahmoud Saleh
Department of Medical Microbiology and Immunology, Faculty of Medicine, Benha University
Tel.: 01141202767
monasaleh898989@gmail.com

ABSTRACT

Background: *The evolution of nosocomial infections by multidrug resisitance (MDR) and extensive drug resistance (XDR) Acinetobacter baumannii and klebsiella pneumoniae are considered a major health problem owing to the relatively limited treatment options. 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:*** *The aim of the current study was 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 suscebtibility 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 suscebtibility 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 aquired 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] .

Carbapenems were previously considered the most active against multidrug-resistant (MDR) gram-negative pathogens and often resorted due to their low toxicity and high efficacy[3]. However, due to the overuse of carbapenems, carbapenem-resistant strains have rapidly emerged in the last decade and most of them are also resistant to at least one agent in most other broad-spectrum antimicrobial categories, these clinical strains are designated as extensively drug resistant (XDR) [4,5].

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 being used as a last choice for infections caused by MDR and XDR organisms, particularly carbapenem-resistant (CR) gram negative bacteria[7]. However, during the last years, colistin resistance increased worldwide especially among *K. pneumoniae* and *A. baumannii* clinical isolates [5]. Colistin resistance has been referred to the loss or modifications of the lipopolysaccharide (LPS) molecule due to mutations in the pmrCAB operon [8, 9, 10, 11].

Tigecycline, a derivative of minocycline, is the first member of the glycylcycline class of antibacterial agents. It inhibits protein translation and impedes amino acid synthesis by reversibly binding to the 30S subunit of the bacterial ribosome [12].

Tigecycline is also considered one of the last choices to treat MDR and XDR bacterial infections. The

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increased use of it led to emerging of its resistance rapidly [13].

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.

METHODOLOGY

This work was done in the Medical Microbiology & Immunology Department, Faculty of Medicine, Benha University in the period 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 males patients, their ages ranged from 20-80 years old.

The present study was approved by Benha University Ethical Committee and written consent was obtained from all patients under study.

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 24h. The growing organisms were identified as *Klebsiella* and *Acinetobacter* by the standard laboratory technique including: Gram staining, colony morphology, 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 pressed gently to ensure good contact. The agar plates were inoculated aerobically at 35°C for16-18h. The discs were Ampicillin/sulbactam (SAM20 = 10/10µg), Tetracycline (TE = 30µg), Ceftriaxone (CRO=30µg), Imipenem (IPM=10µg), ColistinSulphate (CS=10µg), Tigecycline (TGC=30µg).

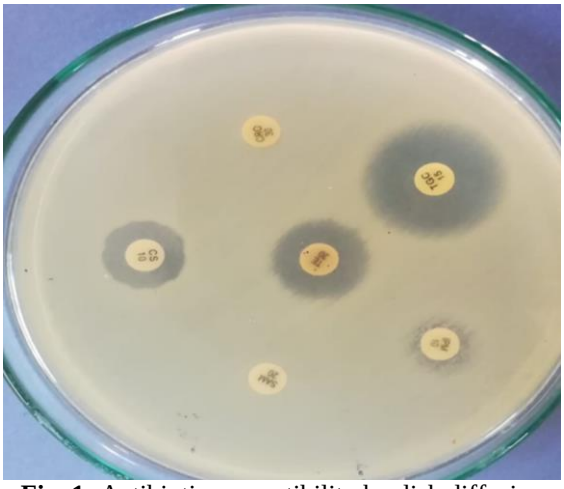

Fig. 1: Antibiotic susceptibility by disk diffusion method.

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°±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).

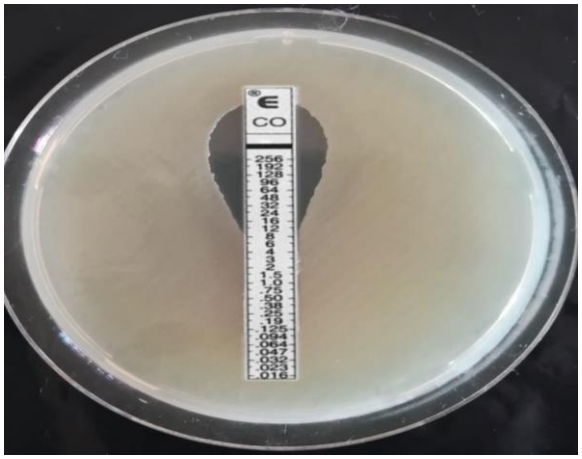

Fig. 2: Colistin E-test

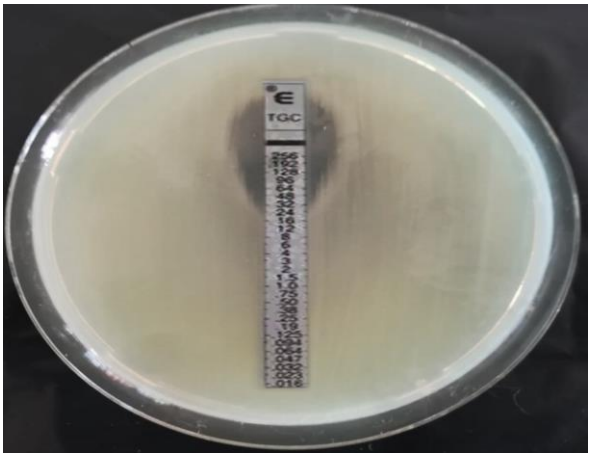

Fig. 3: Tigecycline E-test

Antibiotic susceptibility by VITEK 2 system:

The antibiotic susceptibility testing (AST) for VITEK- 2 System is an automated test methodology based on the MIC technique reported by MacLowry and Marsh and Gerlach. The organism suspension is diluted to a standardized concentration in 0.45% saline before being used to rehydrate the antimicrobial medium within the card. The card was then stacked, sealed, and put into the instrument incubator/reader VITEK-2 system. The instrument monitored the growth of each well in the card over 18 hours for bacteria. At the completion of the incubation cycle, MICs were determined for each antimicrobial contained on the card. The card used was 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 doesn'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).

Data were analyzed by comparing the results produced by the DD, E-test, and VITEK 2 methods for colistin and tigecycline with those produced by the gold standard BD Essential agreement (EA) was defined as the percentage of MICs within ± 1 doubling dilution of the MIC detected by BD. Categorical agreement (CA) was defined as the proportion of isolates classified in the same susceptibility category by BD and the method under estimation. Very major errors (VMEs) denoted a false-susceptible result, major errors (MEs) denoted a false-resistant result, Minor errors (MinEs) denoted susceptible vs. intermediate and intermediate vs. resistant isolates. Acceptable performance was assessed according to criteria detected by the International Organization for Standardization: > 90% for essential or category agreement, < 3% for VMEs or MEs and < 7% for MEs plus MinEs.

RESULTS

Susceptibility to colistin, by BD, 22 % of isolates were colistin resistant, 20% among *K.pneumonaie* and 26.7% among *A. baumnnii*. 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.

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

*NO: number of the strains.

Susceptibility to tigecycline: by BD, 12 % of overall isolates were tigecycline resistant, 11.1 % among *K. pneumonaie* and 13.3 % among *A. baumnnii*. EA and CA were high for E-test (96 & 98% overall) with low MinEs (2%), with no MEs and VMEs, exceeding the acceptable performance rate. On the contrary, VITEK 2 generated an overall low EA and CA rate of 88 & 78 % and high MEs and MinEs (4 &16%). D.D generated low CA (74%) with high MEs and MinEs (8 &18%). 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.

	Method	Susceptible NO(%)	Intermediate NO(%)	Resistant NO(%)	EA NO(%)	CA NO(%)	VME NO(%)	ME NO(%)	Min.E NO(%)
All isolates (NO=50)	BD	40(80)	4(8)	6(12)	-	-	-	-	-
	E-test	40(80)	3(6)	7(14)	48(96)	49(98)	0	0	1(2)
	VITEK 2	34(68)	5(10)	11(22)	44(88)	39(78)	0	2(4)	8(16)
	DD	29(58)	8(16)	13(26)	-	37(74)	0	4(8)	9(18)
K. pneumoniae (NO= 35)	BD	28(80)	3(8.6)	4(11.1)	-	-	-	-	-
	E-test	28(80)	3(8.6)	4(11.4)	33(94.3)	35(100)	0	0	0
	VITEK 2	25(71.4)	4(11.4)	6(17.1)	31(88.6)	28(80)	0	0	6(17.1)
	DD	20(57.1)	6(17.1)	9(25.7)	-	25(71.4)	0	3(8.5)	7(20)
A.baumannii (NO = 15)	BD	12(80)	1(6.7)	2(13.3)	-	-	-	-	-
	E-test	12(80)	0	3(20)	15(100)	14(93.3)	0	0	1(6.7)
	VITEK 2	9(60)	1(6.7)	5(33.3)	13(86.6)	11(73.3)	0	2(13.3)	2(13.3)
	DD	9(60)	2(13.3)	4(26.7)	-	12(80)	0	1(6.7)	2(13.3)

*NO: number of the strains.

DISCUSSION

The increasing emergence of MDR and XDR *A. baumannii* and *K. pneumoniae* led to increase in using colistin and tigecycline as a final treatment option for infections caused by these organisms, particularly carbapenem-resistant gram negative bacteria. However, during the last years, increasing colistin and tigecycline resistance emerged worldwide, especially among *K. pneumoniae* and *A. baumannii* [14].

Choosing an AST method for colistin is challenging due to its poor penetration in the agar medium. BD has been the most favored method of MIC determination by CLSI and FDA, but it requires dedicated staff with good pipetting skills and accurate digital weighing equipment, which may be lacking in multiple current clinical microbiology laboratories .So, rapid and reliable colistin susceptibility testing is needed in the clinical laboratories to allow appropriate therapeutic decision-making. Thus far, few studies have appreciated the

colistin susceptibility methods, displaying controversial results and so, the most perfect one is still challenging[15].

The VITEK2 system is a fully automated device that detects species identification and antimicrobial susceptibility testing for the different clinical isolates, and is recently utilized in many clinical microbiology laboratories worldwide. 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 [16]

Also tigecycline AST is of major importance for the appropriate outcomes. The decreased treatment choices for infections by MDR and XDR bacteria ensure the importance of accurate tigecycline susceptibility methods. The usage of AST has been concerned in controversies due to the reporting of MEs and more specifically, VMEs [16, 17].

The present study reported a high resistance rate of *K. pneumoniae* and *A. baumanii* to different antibiotics i.e. Piperacillin, Ticarcillin/Clavulinic 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. baumanii*, low EA, CA (66.6% & 80%) high MEs and VMEs (13.3% & 6.7%) respectively. Dafopoulou et al.[18], Bakthavatchalam et al.[19] and Hindler and Humphries[20] supported our E-test limitations among total isolates. Chew et al.[17] and Lellouche et al. [21] on *Enterobacteriace* and *A. baumanii* reported low EA and high MEs and VMEs. The poor performance of E-test could be due to the poor diffusion of polymyxin molecules, resulting in a narrow zone of inhibition [17].

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. [18] reported that VITEK 2 exhibited appropriate performance for colistin suscebtibility among *K. pneumonia*e and *A. baumanii*. Lee et al.[22], Dafopoulou et al. [18] and Singhal et al.[23] reported that the CA of the VITEK 2 test was 100%, 90 and 100% respectively with no MEs for *A. baumanii*,

On other hand studies done by Chew et al. [17] and Lellouche et al. [21] on *Enterobactericae* and *A. baumanii* 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% [24, 25, 26].

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% & 88 %) and high MEs and MinEs (4 & 16 %) among *K. pneuomoniae* 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. [29]and Idelevich et al.[32] 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. pneuomoniae*, 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.[29] 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. For colistin, important shortcomings of E-test gradient diffusion and disk diffusion tests, which may result into inappropriate selection of colistin therapy, were probably the most notable observation of this study. Therefore, it is important for the laboratories to be in caution of these results and perform the BD test for colistin susceptibility, especially when colistin therapy is essential. When the BD test can hardly be performed,

the colistin susceptibility should preferably be based on results of automated systems such as VITEK 2.

For Tigecycline, E-test susceptibility method showed the best performance compared to the gold standard BD test. The present study emphasizes a low performance of the VITEK 2 and the DD susceptibility methods, which may falsely decrease the available treatment choices or resulted into inappropriate treatment so need to be confirmed by the BD test, particularly when tigecycline therapy is essential.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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