Are E-test and Vitek2 good choices for tigecycline susceptibility testing when comparing broth microdilution for MDR and XDR *Acinetobacter baumannii*?

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SUMMARY

This study reports the results of antimicrobial susceptibility testing of 10 MDR and 74 XDR *Acinetobacter baumannii* clinical isolates from our hospital routine. We used three different methods: two automated systems (Sensititre and VITEK 2) and one standardized manual method (E-test). Since many published papers refer to *in vitro* tests performed by E-test, the aim of this study was to test if this method is reliable for testing tigecycline. The results obtained show that E-test significantly overestimates the MIC of the broth microdilution (reference test), thus obtaining a significant number of major errors (resistant instead of sensitive). VITEK 2 also shows the same problem, but it is less critical. We therefore conclude that these methods do not seem to be very reliable in the performance of susceptibility testing of MDR and XDR *Acinetobacter baumannii* against tigecycline.

KEY WORDS: Acinetobacter baumannii, MDR, XDR, Tigecycline, Susceptibility testing.

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INTRODUCTION

Acinetobacter baumannii is currently one of the most problematic pathogens, causing above all pneumonia (particularly ventilator-associated ones) and bacteraemia in hospitalized patients, especially in intensive care units (ICUs). *Acinetobacter baumannii* is also a cause of skin and soft-tissue and wounds infections, urinary tract infections and secondary meningitis (Peleg *et al.,* 2008). Over the past years, it has developed resistances to multiple antimicrobial agents (multidrug resistance organism, MDR, or extensively drug resistant, XDR) and so is now difficult to treat. Therefore, *Acinetobacter* spp. strains have emerged in recent years as one of the most problematic pathogens to eradicate, using available antimicrobial agents when possible (Marchaim *et al.,* 2007).

In several cases, colonized or infected patients spread *Acinetobacter baumannii* to other patients in the same ward, primarily due to hand contact by hospital staff, thus becoming frequently colonized, for a variable time that can be very long (Marchaim *et al.,* 2007).

Agents potentially effective against these MDRs include carbapenems, aminoglycosides (amikacin or gentamycin), tetracyclines (minocycline or doxycycline) and sulbactam. However, combined resistance to all of the above agents has been increasingly reported in recent years (Livermore *et al.,* 2010). Moreover, MDR and XDR *Acinetobacter baumannii* strains remain susceptible to polymyxins (colistin and polymyxin

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B), which have been re-introduced after many years in clinical practice. A standardized international terminology of MDR and XDR *Acinetobacter baumanni* was created: according to Magiorakos *et al*. (2012), we considered MDR the strains non-susceptible to ≥1 agent in ≥3 antimicrobial categories (aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins + β-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins + β-lactamase inhibitors, polymyxins, tetracyclines), and XDR the strains non-susceptible to ≥1 agent in all but ≤2 categories.

Tigecycline was licensed by the European Medicines Agency (EMEA) for parenteral use in complicated skin and soft tissue infections and complicated intra-abdominal infections caused by *Enterobacteriaceae* (excluding *Proteus* spp., which are resistant to tigecycline), enterococci, staphylococci and streptococci. It seems to be a promising agent for the treatment of MDR and XDR *Acinetobacter* infections (Seifert *et al.,* 2006).

CLSI (Clinical and Laboratory Standards Institute) and EUCAST (Committee to Harmonize Antimicrobial Breakpoints, organized by ESCMID, ECDC and European national breakpoint committees) have their own breakpoint susceptibility for *Acinetobacter baumannii,* but they do not have one for tigecycline. Several authors have used interpretative criteria for tigecycline MICs as approved by the United States Food and Drug Administration (FDA) for *Enterobacteriaceae* (sensitive ≤2 mg/L and resistant ≥8) (Seifert *et al.,* 2006; Navon-Venezia *et al.,* 2007; Eser *et al.,* 2008; Kulah *et al.,* 2009; Al-Sweih *et al.,* 2011). EUCAST defines the Epidemiological Cut-Off (ECOFF) as the MIC that separates microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to every agent. This combination for *A. baumannii* and tigecycline is ≤1 mg/L and so strains with MIC equal to or below this value can be considered sensitive. So, we considered susceptible all the strains that have MICs ≤1mg/L (wild type), and not-susceptible (from now on called resistant) all the strains that have MICs ≥2 mg/L (not wild type) to tigecycline (Seifert *et al.,* 2006, Navon-Venezia *et al.,* 2007; Zarkotou *et al.,* 2012).

The aim of this study was to define and compare the performances of three different methods for the determination of *in vitro* susceptibility tests to *Acinetobacter baumannii* versus tigecycline. In particular, we wanted to determine if the E-test, used for *in vitro* tests in many studies (Eser *et al.,* 2008; Pillar *et al.,* 2008; Kulah *et al.,* 2009; Al-Sweih *et al.,* 2011), is a good choice for the determination of the sensitivity of the strains to this antibiotic.

MATERIALS AND METHODS

Bacterial isolates

The *in vitro* activity of tigecycline was evaluated against 10 MDR and 74 XDR *Acinetobacter baumannii* clinical isolates. Isolates were collected in the Ospedale dell'Angelo, Mestre (Venice, Italy) from January 2009 to June 2012 and were isolated from lower respiratory tract (27; 32.1%), pus (22; 26.2%), urine (17; 20.2%), blood culture and central venous catheter (12; 14.3%), biological fluids (4; 4.8%) and stools (2; 2.4%). The first number in parenthesis represents the number of the specific isolates examined while the second number represents the percentage of the total number of isolates examined. Only one isolate per patient was included in the study. The strains were included in the study only if there were no other strains of *Acinetobacter baumannii* with the same biochemical pattern in the isolation ward.

Species identification was determined by Vitek 2 automated system (bioMerieux, Marcy l'Etoile, France).

Isolates were all identified as having a MDR or XDR phenotype based on the definition that all of them exhibited resistance to three or more groups of antibiotics. Our strains showed resistance to ciprofloxacin, levofloxacin, piperacillin, piperacillin plus tazobactam, ticarcillin plus clavulanate, trimethoprim/sulphametoxazole in 100% of isolates; ceftazidime and meropenem in 98.8% of isolates (1.2% shows intermediate susceptibility); amikacin in 94.1% (4.7% susceptible and 1.2% intermediate); imipenem in 91.6% (2.4% susceptible and 6% intermediate); aztreonam in 86.9% (13.1% intermediate); ampicillin plus sulbactam in 84.6% (7.1% susceptible and 8.3 intermediate). The strains were

all susceptible to colistin (100%) and in 95.2% to tigecycline (4.8% resistant).

Sensititre

Sensititre antimicrobial susceptibility tests were performed with MIC plates ITNF1F (Trek Diagnostics Systems, Cleveland, OH, USA). The concentrations of the agent in the plates were: 0.03; 0.06; 0.12; 0.25; 0.5; 1 and 2 mg/L.

For each strain, 10 µl of suspension of 0.5 McFarland were added to Sensititre® cation adjusted Mueller-Hinton broth with TES buffer (Trek Diagnostics Systems); the automatic inoculator dispensed 50 µl of this broth into each well. The plates were covered with an adhesive seal, incubated in ARIS (incubator and reader) at 35° C for 18-24 h and analyzed with SWIN™ software.

Vitek2

AST-N097 cards (bioMérieux, Inc., Durham, NC, USA) were used for VITEK 2 antimicrobial susceptibility tests. The concentrations of tigecycline in the cards were: 0.5; 0.75; 1; 2; 4 and 8 mg/L. The results were analyzed with AES software (version WSVT2-R05.01).

E-test

Tigecycline MIC was determined by E-test in Mueller-Hinton agar. An inoculum suspension with a turbidity equivalent to 0.5 McFarland standard was prepared by suspending well-isolated colonies in 0.9% saline. A sterile cotton swab before was dipped into the inoculum suspension and then was used to streak the agar surface.

E-test susceptibility tests were performed in accordance with manufacturer's (bioMerieux, Marcy l'Etoile, France) instructions on Mueller-Hinton Agar plates. The strips were stored at 4° C and brought to room temperature 10 minutes before using them. Mueller-Hinton agar plates (bioMerieux, Marcy l'Etoile, France) were inoculated as in the disk-diffusion method and were incubated for 18-24 h in an aerobic atmosphere at 37°C. The antibiotic concentration range of the E-test was 0.016-256 mg/L. The MIC values were read at the point of 80% inhibition protocol according to the instructions for a bacteriostatic antimicrobial provided by the manufacturer.

Acinetobacter baumannii strain ATCC 19606 and *Escherichia coli* strain ATCC 25922 were used as quality control for all methods.

Statistical analysis

The automated methods of VITEK 2 and Sensititre test using 6 and 7 tigecycline dilutions respectively, gave MIC values only in some ranges. It was necessary to make several changes in order to have an appropriate comparison to the E-test results. The MIC values obtained from three antimicrobial susceptibility methods were shifted to the higher level to obtain the same type of dilutions for all (for example, 0.016 and 0.023 mg/L from E-test became 0.03 mg/L).

Simple linear correlation, paired t-test and Bland-Altman test were performed to compare the three different tests (Bland *et al.*, 1986). Moreover, the comparisons were supplemented with 2x2 tables and Fisher exact test using MIC ≥2 mg/L for classifying strains as 'resistant'. All tests were considered significant with a p value < 0.05. JMP-SAS software was used for the analysis.

RESULTS

74 *Acinetobacter baumannii* strains were XDR and only 10 were MDR. MIC_{50} and MIC_{90} values of the three tests are presented in Table 1.

Sensititre gave mean values of 0.50±0.45 mg/L, median 0.25 (interquartile range 0.25-0.50) (full range 0.12-2.00), Vitek2 mean values were 2.64±2.98 mg/L, median 1.00 (interquartile range 0.50-3.5) (full range 0.50-8.00), E-Test mean values were 13.9±42.9 mg/L, median 2.00 (interquartile range 2.00-4.00) (full range 0.25-256.00). Of the 84 tested samples, only 4 MIC values (5%) were ≥ 2.00 mg/L with Sensititre, 31 MIC values (37%) were ≥2.00 mg/L with Vitek2 and 75 MIC values (89%) were ≥2.00 mg/L with E-Test.

Table 2 shows MICs of tigecycline obtained with the three methods.

The correlation matrix between the three methods showed good results for the relationship between Sensititre and Vitek2 but low coefficients for E-Test (Table 3).

The Bland-Altman test for the agreement be-

tween Sensititre and Vitek2 shows a difference of 2.14 mg/L between the tests with 95% limits 1.57-2.70, a t-test p<0.001 and an evident trend of error amplification with higher mean values of MIC.

The Bland-Altman test for the agreement between Sensititre and E-Test shows a difference of 13.37 mg/L between the tests with 95% limits of 4.10-22.6, a t-test p=0.005 and an evident trend of error amplification with higher mean values of MIC (Figure 1).

The comparison between the *in vitro* sensitivity and resistance to tigecycline using the three test used (Sensititre vs Vitek2 and vs E-test) is shown in Table 4.

Classifying the MIC values given by the three tests ≥2.00 mg/L as resistant (R) and <2.00 mg/L as sensitive or wild type (S), enabled us to compare the information from a clinical practice point of view, considering all sensitivity results that are resistant at the reference test as major

TABLE 1 - *Percentage of susceptible and resistant strains and MIC50 and MIC90 of Tigecycline using the three methods.*

	% of isolates		*MIC (mg/L)*	
	Susceptible	*Resistant*	*50%*	*90%*
Sensititre	95,2	4,8	0,25	1,00
Vitek2	63,0	37,0	1,00	8,00
Etest	10,7	89,3	2,00	16,00

TABLE 2 - *MICs of Tigecycline using the three methods.*

MIC (mg/L)	*Sensititre*	*Vitek2*	*E-test*
0.12	11		
0.25	39		2
0.50	14	22	
1.00	16	31	7
2.00	4	10	41
4.00		2	15
8.00		19	1
16.00			13
32.00			1
128.00			2
256.00			2

TABLE 3 - *The correlation matrix between the three methods used.*

	MIC Sensititre	*MIC Vitek2*	*MIC E-Test*
MIC Sensititre	1.0000	0.8830	0.3376
MIC Vitek2	0.8830	1.0000	0.4902
MIC E-Test	0.3376	0.4902	1.0000

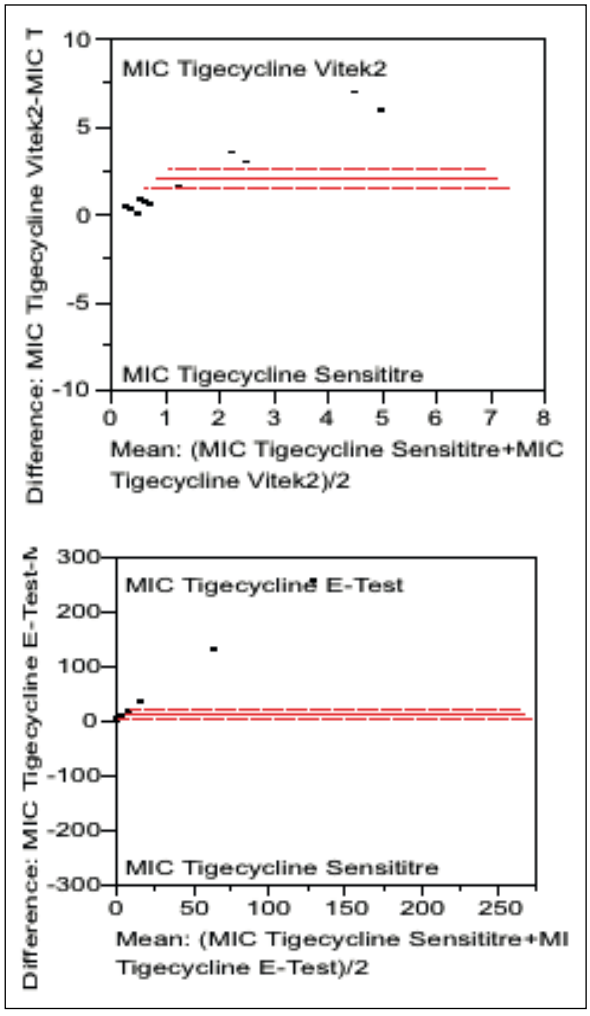

FIGURE 1 - *Bland Altman test between Sensititre and Vitek2 (top panel) / E-Test (above).*

TABLE 4 - *Comparison between the in vitro sensitivity and resistance to Tigecycline using Vitek2, E-test and Sensititre.*

	Sensititre R N° of strains (% of total)	*Sensititre S N° of strains (% of total)*
Vitek2 R	4 (4,76)	27 (32,14)
Vitek2 S	0 (0,00)	53 (63,10)
E-test R	4 (4,76)	71 (84,52)
E-test S	0 (0,00)	9 (10,71)

errors. Considering Sensititre as reference, 0 cases R at this test (4 strains with MIC as 2.00 mg/L) are S with Vitek2 and also with E-Test.

DISCUSSION

Multidrug-resistant *Acinetobacter baumannii* are increasing all over the world (Nordman *et al*, 2011). Carbapenem can become a powerful selector for MDR or XDR strains being a second line therapeutic option in infections caused by this microorganism.

Therapeutic options currently available to treat MDR and XDR *Acinetobacter baumannii* infections are few. Often polymyxins cannot be used because of their toxicity.

However, one question is the determination of the *in vitro* susceptibility of tigecycline against *Acinetobacter baumannii*. In fact, therapy should be based on the results of a "well" performed antimicrobial susceptibility test. Several authors (Seifert *et al.,* 2006; Navon-Venezia *et al.,* 2007; Al-Sweih *et al.,* 2011; Zarkotou *et al*., 2012) reported those of the FDA for *Enterobacteriaceae* (sensible ≤2.00 mg/L, resistant ≥8.00 mg/L). According to Canigia *et al.,* 2009, we do not consider disc diffusion a good method for testing tigecycline susceptibility versus *Acinetobacter* spp. In our experience, we considered the ECOFF of the wild type strains proposed by EUCAST, set at ≤1.00 mg/L. All strains with MIC ≤1.00 mg/L are considered to be lacking mutations that can lead to resistance, without natural ones and thus susceptible to tigecycline.

The second and perhaps most important problem is to understand if all the methods used to determine the MIC of tigecycline versus *Acinetobacter baumannii* give the same results so they can be used interchangeably. This study compared the results obtained using three different methods: broth microdilution (Sensititre), Vitek and E-test. Many authors have used E-test for susceptibility testing of *Acinetobacter baumannii* versus tigecycline (Eser *et al.,* 2008; Pillar *et al.,* 2008; Kulah *et al.,* 2009; Al-Sweih *et al.,* 2011;). Our results show that the MIC_{50} and MIC_{90} differ substantially if we use the different methods. In particular, considering that we set the susceptibility breakpoint for all values at ≤1.00 mg/L, the MIC_{90} for only broth microdilution was within these limits, while Vitek2 and especially E-test were higher. This raises questions about what Al-Sweih *et al.,* 2011 reported on the appearance of resistant strains when tested by E-test. In fact, even in our series, 71 of the 84 strains were resistant using E-test and sensitive with Sensititre. We also found some differences when comparing the Sensititre and Vitek2, with 27 strains susceptible with Sensititre and resistant with Vitek2. If we consider broth microdilution as the reference method, it is clear that with both Vitek2 and more so with E-test, the percentage of major errors (susceptible strains considered resistant) is very significant. The cause of elevated MIC from E-test cannot be totally attributable to the use of a Muller Hinton medium supplemented with manganese ions, already proved to influence the results of the test for antibiotic sensitivity (Fernandez-Mazarrasa C. *et al.,* 2007), since the medium we used does not contain this element.

Our conclusions are that extreme caution must be taken when interpreting MIC data obtained by the use of Vitek2 and more E-test strips of Tigecycline when testing strains of MDR and XDR *Acinetobacter baumannii*. Subsequently, the results obtained by these two methods should always be confirmed by broth microdilution.

Therefore we suggest using a manual or semiautomatic broth microdilution method (eg. Sensititre, Trek Diagnostics Systems) in routine clinical practice when it is useful to give the physician the tigecycline MIC in infections caused by MDR and XDR *Acinetobacter baumannii*.

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