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COMPARISON OF TWO DIFFERENT METHODS FOR TIGECYCLINE SUSCEPTIBILITY TESTING IN *ACINETOBACTER BAUMANNII*

Branka Bedenić[1,2], Gordana Cavrić[3], Mirna Vranić-Ladavac[4], Nada Barišić[5], Natalie Karčić[4], Tatjana Tot[5], Aleksandra Presečki-Stanko[2], Amarela Lukić-Grlić[1,6], Sonja Frančula-Zaninović[7] and Katherina Bernadette Sreter[8]

[1]Department of Microbiology, School of Medicine, University of Zagreb, Zagreb, Croatia; [2]Department of Clinical and Molecular Microbiology, Zagreb University Hospital Centre, Zagreb, Croatia; [3]Department of Intensive Care, Merkur University Hospital, Zagreb, Croatia; [4]Department of Microbiology, Public Health Institute of Istria County, Pula, Croatia; [5]Department of Microbiology, Karlovac General Hospital, Karlovac, Croatia; [6]Department of Clinical Microbiology, Zagreb Children's Hospital, Zagreb, Croatia; [7]Zagreb Health Centre, Zagreb, Croatia; [8]Department of Clinical Immunology, Pulmonology and Rheumatology, Sestre milosrdnice University Hospital Centre, Zagreb, Croatia

SUMMARY – Tigecycline susceptibility testing (TST) presents a tremendous challenge for clinical microbiologists. Previous studies have shown that the Epsilometer test (E-test) and Vitek 2 automated system significantly overestimate the minimum inhibitory concentrations for tigecycline resistance compared to the broth microdilution method (BMM). This leads to very major errors or false susceptibility (i.e. the isolate is called susceptible when it is actually resistant). The aim of this study was to compare E-test against BMM for TST in carbapenem-resistant and carbapenem-susceptible *Acinetobacter (A.) baumannii* and to analyze changes in tigecycline susceptibility between two time periods (2009-2012 and 2013-2014), with BMM as the gold standard. Using the EUCAST criteria, the rate of resistance to tigecycline for the OXA-23 MBL-positive, OXA-23 MBL-negative and carbapenemase-negative strains for BMM was 54.5% (6/11), 29.4% (5/17) and 2.7% (1/37), respectively; the OXA-24/40 and OXA-58 producing organisms did not exhibit any resistance. With E-test, all OXA-23 MBL-positive organisms (11/11), 23.5% (4/17) of OXA-23 MBL-negative, and 4.1% of OXA-24/40 (3/74) strains displayed tigecycline resistance; there were no resistant strains among the OXA-58 and carbapenemase-negative isolates. Resistance emerged in the bacterial isolates from 2013 to 2014. Although tigecycline does not display cross-resistance, the highest rates of resistant *A. baumannii* isolates were observed among those producing VIM MBL, regardless of the testing method. These findings suggest that the commercial E-test does not provide reliable results for TST of *A. baumannii*. Further confirmation with the dilution method should be recommended, particularly in cases of serious infections.

Key words: *Tigecycline; Disk diffusion, antimicrobial tests; Microbial sensitivity tests; Acinetobacter baumannii; Drug resistance, microbial*

Introduction

Acquired carbapenem resistance is an emerging problem in *Acinetobacter (A.) baumannii* due to the production of acquired carbapenemases of class A

Correspondence to: *Prof. Branka Bedenić, MD, PhD*, Department of Clinical and Molecular Microbiology, Zagreb University Hospital Centre, Kišpatićeva 12, HR-10000 Zagreb, Croatia
E-mail: branka.bedenic@kbc-zagreb.hr, bbedenic@mef.hr
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(KPC)[1], class B metallo-β-lactamases (MBLs) of the IMP, VIM, SIM and NDM families[2-6], and class D carbapenem-hydrolyzing oxacillinases (OXA-23-, OXA-40-, OXA-58-, OXA-143- and OXA-238-like) [CHDL][7-12]. Carbapenem-resistant *A. baumannii* (CRAB) isolates have been reported worldwide[13]. Carbapenem resistance in *A. baumannii* is a growing concern in Croatia and neighboring countries[14-20]. In addition to the hospital setting, CRAB has also been identified in environmental samples in Croatia[21-23]. CRAB isolates are frequently associated with serious infections such as ventilator-associated pneumonia, septicemia and urinary tract infections, specifically in intensive care units[24-25]. They are often a cause of wound, skin and soft tissue infections, and secondary meningitis[25].

Tigecycline and colistin are often last-resort antibiotics for the treatment of infections associated with carbapenemase-producing organisms. Tigecycline, the first semisynthetic glycocycline, is a minocycline derivative that overcomes major tetracycline resistance mechanisms[26]. However, tigecycline resistance has also emerged[27]. Therapeutic decisions often rely on appropriate susceptibility testing. The issue is that tigecycline susceptibility testing (TST) remains a major challenge for clinical microbiologists. Thus far, there are no clear guidelines established by either the Clinical and Laboratory Standards Institute (CLSI)[28] or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for TST of *A. baumannii*.

In most studies, the breakpoints applied for Enterobacteriaecae include the susceptibility breakpoint of ≤1 mg/L and the resistance breakpoint of ≥4 mg/L. Disk-diffusion test is not appropriate for susceptibility testing for tigecycline. Previous studies have shown that the Epsilometer test (E-test) and Vitek 2 system significantly overestimate the minimum inhibitory concentrations (MICs) of tigecycline compared to the broth microdilution method (BMM), leading to very major errors (i.e. false susceptibility)[29,30]. The aim of this study was to compare two different methods for TST (E-test and BMM) in carbapenem-resistant and carbapenem-susceptible *A. baumannii* and to analyze dynamic changes in tigecycline susceptibility between two collection periods (2009-2012 and 2013-2014). The first period (2009-2012) was chosen because it was in that time-frame that the first carbapenem-resistant isolates were identified in both countries (Republic of Croatia and Republic of Bosnia and Herzegovina). During the second period (2013-2014), it was observed that tigecycline resistance had emerged and this prompted further evaluation of its prevalence and TST problems. In this study, BMM was considered as the gold standard for TST.

Material and Methods

Bacteria

During the two collection periods, a total of 154 bacterial isolates were obtained. Within the scope of this multicentre study, the isolates from 2009 to 2012 were retrieved from 13 different hospital centers in Croatia and from the Mostar General Hospital in Bosnia and Herzegovina. The isolates from the second period (2013-2014) were collected in two centers in Croatia, the Pula General Hospital and Godan Nursing Home in Zagreb. Bacterial strains were identified by conventional biochemical testing (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF MS) and verified by polymerase chain reaction (PCR) for $bla_{\text{OXA-51}}$ gene. Molecular characterization of carbapenem resistance was performed as in previous studies[16-20].

Antibiotic susceptibility testing

The isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR) according to Magiorakos *et al.*[31]. Susceptibility to tigecycline was determined by BMM and E-test. Antimicrobial susceptibility was confirmed by BMM in Mueller-Hinton broth in 96-well microtiter plates according to the CLSI guidelines[28]. *Pseudomonas aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606 were used as quality control strains. Since CLSI does not have interpretative criteria for TST for *A. baumannii*, resistance rates were calculated according to the EUCAST criteria for Enterobacteriaceae[32] or the U. S. Food and Drug Administration (FDA) criteria[33], with resistance breakpoints of >2 or ≥8 mg/L, respectively.

Interpretation of data

Categorical agreement (CA) was defined as the percentage of isolates recorded in the same susceptibility category by BMM and E-test as defined previously

Table 1. Number and percentage of resistant isolates, MIC_{50} and MIC_{90} among A. baumannii isolates with different carbapenemases with two different testing methods

Type of oxacillinase	Number and % of resistant strains according to EUCAST with BMM	Number and % of resistant strains according to FDA with BMM	Number and % of resistant strains according to EUCAST with E-test	Number and % of resistant strains according to FDA with E-test	MIC range (BMM) mg/L	MIC_{50} (BMM) mg/L	MIC_{90} (BMM) mg/L	MIC range (E-test) mg/L	MIC_{50} (E-test) mg/L	MIC_{90} (E-test) mg/L
OXA-23 MBL positive	6/11 (54.5%)	3/11 (27.3%)	11/11 (100%)	0/11 (0%)	1-8	4	8	4-4	4	4
OXA-23 MBL negative	5/17 (29.4%)	0/17 (0%)	4/17 (23.5%)	0/17 (0%)	0.25-4	2	4	1-4	2	4
OXA-24/40	0/74 (0%)	0/74 (0%)	3/74 (4.1%)	0/74 (0%)	0.5-2	1	2	0.5-4	2	3
OXA-58	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0.5-2	1	2	0.5-2	2	2
Carbapenemase negative	1/37 (2.7%)	0/37 (0%)	0/37 (0%)	0/37 (0%)	0.06-4	1	4	0.25-2	2	2

EUCAST = European Committee on Antimicrobial Susceptibility Testing; FDA = U. S. Food and Drug Administration; BMM = broth microdilution method; MIC = minimum inhibitory concentration; MIC_{50} = antibiotic concentration which inhibits 50% of the strains; MIC_{90} = antibiotic concentration which inhibits 90% of the strains; E-test = epsilometer test

by Zarkotou *et al.*[30]. Category discrepancies were grouped as follows: (i) very major errors (VME) in cases where BMM indicated resistance and the comparative method indicated susceptibility; (ii) major errors (ME) when an isolate was categorized as susceptible by BMM and resistant by the comparative method; and (iii) minor errors (mE) when there was one interpretation category difference between BMM and the comparative method. Essential agreement (EA) was considered to be the percentage of MICs within 1 doubling dilution of the MIC determined by BMM[30].

Results

Isolates with acquired oxacillinases were labeled MDR or XDR, as described by Magiorakos *et al.*[31]. When a MIC breakpoint of >2 mg/L was applied according to the EUCAST criteria for defining resistance to tigecycline[32], the rate of resistance for BMM was 54.5% (6/11) for OXA-23 MBL-positive, 29.4% (5/17) for OXA-23 MBL-negative, and 2.7% (1/37) for carbapenemase-negative strains. The OXA-24/40- and OXA-58-producing organisms did not exhibit any resistance. In contrast, in E-test, all OXA-23 MBL-positive organisms (11/11), 23.5% (4/17) of OXA-23 MBL-negative, and 4.1% (3/74) of OXA-24/40 strains showed resistance to tigecycline, as displayed in Table 1. There were no resistant strains among the OXA-58 and carbapenemase-negative isolates. Using the FDA criteria with a resistance breakpoint of ≥8 mg/L[33], no resistant isolates were detected, regardless of the testing method. The strains collected from 2009 to 2012 exhibited full susceptibility to tigecycline. However, resistance emerged in those obtained during the 2013-2014 period.

The MIC_{90} ranged from 2 mg/L (OXA-24/40 and OXA-58) to 8 mg/L (OXA-23 MBL positive) with BMM and from 2 mg/L (OXA-24/40, OXA-23 MBL-negative and OXA-58) to 4 mg/L (OXA-23 MBL positive) in E-test. The MIC_{50} varied between 1 and 4 mg/L with BMM and between 2 and 4 mg/L in E-test (Table 1).

When BMM was considered the gold standard for antibiotic susceptibility testing, 5 (45.5%) MBL-positive and 8 (10.7%) OXA-24/40 isolates were noted as resistant instead of susceptible, demonstrating a ME of E-test (Table 2). VME (i.e. resistant strain being classified as susceptible) was identified in only one

Table 2. Rate of errors in E-test compared to broth microdilution method

Type of oxacillinase	% of VME	% of ME	% of mE	% of EA	% of CA
OXA-23 MBL- positive	0/11 (0%)	5/11 (45.5%)	5/11 (45.5%)	9/11 (81.8%)	6/11 (54.5%)
OXA-23 MBL- negative	0/17 (0%)	0/17 (0%)	5/17 (29.4%)	15/17 (88.2%)	12/17 (70.6%)
OXA-24/40	0/75 (0%)	8/75 (10.7%)	44/75 (58.7%)	71/75 (94.7%)	44/75 (58.7%)
OXA-58	0/15 (0%)	0/15 (0%)	5/15 (33.3%)	14/15 (93.3%)	10/15 (66.7%)
Carbapenemase negative	1/37 (2.7%)	0/37 (0%)	10/37 (27.0%)	32/37 (86.5%)	10/37 (27.0%)

VME = very major error; ME = major error; mE = minor error; EA = essential agreement; CA = categorical agreement

(2.7%) of the carbapenemase-negative strains (Table 2). The rates of mEs were 58.7%, 45.5%, 33.3%, 29.4% and 27.0% for OXA-24/40, MBL, OXA-58, OXA-23 MBL-negative and carbapenemase-negative isolates, respectively. The highest rate of EA was observed for OXA-24/40 (94.7%), followed by OXA-58 (93.3%), OXA-23 MBL-negative (88.2%), carbapenemase-negative (86.5%), and OXA-23 MBL-positive (81.8%) strains (Table 2). The CA was highest in OXA-23 MBL-negative strains (70.6%) and lowest in carbapenemase-negative strains (27.0%).

Discussion

Although tigecycline does not display cross-resistance, the highest rates of resistance were observed among the VIM MBL-producing isolates, regardless of the testing method. Emergence of tigecycline resistance was detected for isolates from the 2013-2014 collection period, whereas those from 2009 to 2012 were fully susceptible. Tigecycline testing by E-test produced higher MICs, yielding ME in 45.5% of MBL-positive isolates, while VME was detected in only one carbapenemase-negative strain. The rates of EA of approximately 80% to 90% and CA of 60% to 70% were similar to those found by Zarkotou *et al*.[30]. There are no published studies so far on the accuracy of particular tests in isolates with different carbapenem-resistance mechanisms.

According to the results of this study, E-test did not provide reliable results. Hence, these should be substantiated by the dilution method. This is especially important in case of severe infections. Although considered the gold standard, BMM is laborious, time-consuming and requires educated staff. In addition, these findings confirm the elevated MICs of tigecycline by E-test compared to BMM, as previously detailed by other authors. However, the discrepancies between these two methods were less pronounced in our study than in the published literature, where the MICs of BMM were overestimated two- to three-fold by the E-test[30].

Like the present study, false-resistant outcomes (i.e. MEs) have also been reported in previous studies[29,30]. The explanation provided by Marchaim *et al*. is that E-test detects heteroresistance that is very common in *A. baumannii* and cannot be identified by broth methods[34]. The phenomenon of increased MICs by E-test is unique only for *A. baumannii* and has not been detected in Enterobacteriaceae[34]. The resistance to tigecycline was predominantly associated with VIM-producing organisms. Nonetheless, a limitation of this study was that the MBL-positive isolates from the nursing home in Zagreb belonged to a single clone. It is difficult to explain the reason for cross-resistance in MBL-positive isolates since MBLs are encoded on mobile genetic elements, whereas tigecycline resistance is due to hyperexpression of efflux pumps. A possible explanation may be that the carbapenemase-producing organisms have a greater ability to acquire other resistance traits as well.

Conclusions

Given that BMM is time-consuming and necessitates educated staff, it is not routinely performed in most laboratories. Instead, either the E-test or Vitek 2 is preferred. Clinicians and laboratory personnel alike should be made aware of the discordances between E-test and other microbial sensitivity testing methods, particularly in critically ill patients. Therefore, according to our results, BMM should be recommended for TST of *A. baumannii*.

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Sažetak

USPOREDBA DVIJU RAZLIČITIH METODA ZA TESTIRANJE OSJETLJIVOSTI NA TIGECIKLIN U *ACINETOBACTER BAUMANNII*

B. Bedenić, G. Cavrić, M. Vranić-Ladavac, N. Barišić, N. Karčić, T. Tot, A. Presečki-Stanko, A. Lukić-Grlić, S. Frančula-Zaninović i *K.B. Sreter*

Testiranje osjetljivosti na tigeciklin (TST) je velik izazov za kliničke mikrobiologe. Prethodna istraživanja su pokazala da E-test i Vitek 2 daju veće vrijednosti minimalne inhibitorne koncentracije tigeciklina u odnosu na dilucijsku metodu, što uzrokuje vrlo veliku grešku (engl. *very major error*, što znači da je rezistentan izolat proglašen osjetljivim). Cilj istraživanja bio je usporediti dvije metode za testiranje osjetljivosti na tigeciklin (E-test i bujonska dilucijska metoda) u karbapenem osjetljivim i karbapenem rezistentnim izolatima *Acinetobacter (A.) baumannii* s različitim tipovima karbapenem-hidrolizirajućih oksacilinazama i analizirati promjenu u stopama osjetljivosti na tigeciklin u dva razdoblja istraživanja (2009.-2012. i 2013.-2014.). Dilucija u bujonu je bila referentna metoda. Testiranje osjetljivosti na tigeciklin je provedeno E-testom i bujonskom mikrodilucijskom metodom. Prema kriterijima EUCAST-a stopa rezistencije bila je 54,5% (6/11) za OXA-23 MBL-pozitivne sojeve, 29,4% (5/17) za OXA-23 MBL-negativne sojeve i 2,7% (1/37) za karbapenemaza-negativne sojeve uz bujonsku mikrodilucijsku metodu. OXA-24/40 i OXA-58 producirajući sojevi nisu iskazivali rezistenciju. E-testom su svi OXA-23 MBL pozitivni organizmi (11/11), 23,5% (4/17) OXA-23 MBL negativnih i 4,1% OXA-24/40 (3/74) pokazivali rezistenciju na tigeciklin. Svi OXA-58 pozitivni i karbapenemaza-negativni sojevi su bili osjetljivi na tigeciklin u E-testu. Rezistencija na tigeciklin se pojavila u razdoblju od 2013. do 2014. godine. Iako tigeciklin ne pokazuje križnu rezistenciju s drugim antibioticima najviše stope rezistencije su zapažene među VIM-pozitivnim izolatima bez obzira na metodu testiranja. Prema rezultatima našega istraživanja komercijalni E-test ne daje pouzdane rezultate TST u *A. baumannii*. Potrebna je potvrda dilucijskom metodom, osobito kod teških infekcija.

Ključne riječi: *Tigeciklin; Disk difuzija, antimikrobni testovi; Mikrobi, testovi osjetljivosti; Acinetobacter baumannii; Lijekovi, rezistencija, bakterijska*