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Original Scientific Paper

INVESTIGATION OF TIGECYCLINE SUSCEPTIBILITY OF MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII* ISOLATES BY DISC DIFFUSION, AGAR GRADIENT AND BROTH MICRODILUTION TESTS

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SUMMARY – The use of tigecycline is becoming increasingly important because of the high levels of antibiotic resistance in *Acinetobacter baumannii* (*A. baumannii*) isolates. In this prospective study, multidrug-resistant *A. baumannii* isolates were obtained from various tissue and fluid samples of patients admitted to or treated at various departments and tested in Laboratory of Microbiology, Duzce University Medical Faculty between January 2013 and December 2015. Tigecycline resistance in multidrug-resistant *A. baumannii* isolates were analyzed using the disc diffusion test (DDT), agar gradient test (AGT), and gold standard test [broth microdilution test (BMT)]. *A. baumannii* isolates resistant to multiple drugs were included in the study (N=94). Using the BMT method, 89 (95%), 4 (4%) and 1 (1%) *A. baumannii* isolates were determined as tigecycline susceptible, intermediate and resistant isolates, respectively. Using the Food and Drug Administration criteria, the rates of major error (ME), minor error (mE) and categorical agreement (CA) for DDT were 26%, 67% and 9%, respectively. In contrast, for AGT, the rates of ME, mE and CA were 0%, 4%, 95%, respectively. Tigecycline resistance as assessed by BMT showed no increase between 2013 and 2015. Accordingly, isolates found to be resistant or intermediate by DDT should be confirmed by BMT. Due to the ease of application, AGT is a safe method of detecting susceptibility.

Key words: *Acinetobacter baumannii; Agar gradient test; Broth microdilution test; Disc diffusion test; Multidrug-resistance; Tigecycline*

Introduction

Acinetobacter species are among the leading causes of nosocomial infections in recent years. *Acinetobacter (A.) baumannii* are immobilized, non-fermentative, oxidase negative, gram-labile coccobacilli that cause serious infections such as ventilator-associated pneumonia, meningitis, endocarditis, and bloodstream, urinary tract and wound infections. Development of resistance to many antibiotics, including the carbapenem group, has contributed to treatment failure. Multidrug-resistance, defined as resistance to typical molecules from at least three classes of antibiotics, is frequently and especially found in *A. baumannii* isolates[1]. Therefore, drug regimens containing combinations of colistin and tigecycline are used in the treatment of multidrug-resistant infections[2,3].

Tigecycline is a relatively new, broad spectrum, semisynthetic glycylcycline derived from minocycline with *in vitro* activity against multidrug-resistant *A.*

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baumannii, many gram-positive and gram-negative facultative aerobes, and anaerobic bacteria[4,5]. The disc diffusion test (DDT), agar gradient test (AGT) and broth microdilution test (BMT) are typically used to determine the susceptibility of bacterial isolates to tigecycline[1].

This study aimed to detect the rate of tigecycline resistance in *A. baumannii* isolates that are resistant to multiple drugs and the change of resistance rate over years. Further, we also wanted to assess conformity among tigecycline susceptibility profiles generated by DDT, AGT and BMT as the gold standard test, to precisely determine sensitivity.

Material and Methods

In this prospective study, multidrug-resistant *A. baumannii* isolates were obtained from various tissue and fluid samples of patients admitted to or treated at the various departments, and tested in Laboratory of Microbiology, Düzce University Medical Faculty between January 2013 and December 2015. *A. baumannii* isolates were identified using conventional microbiological methods on an automated bacterial identification system (Vitek 2, bioMerieux, France). Antibiotic susceptibility of *A. baumannii* isolates to tigecycline was tested using the methods described below.

Disc diffusion test

Bacterial suspensions prepared to 0.5 McFarland turbidity units were inoculated onto MH agar (Oxoid, UK) and a 15-µg tigecycline disc (Bioanalyze, Turkey) was placed on the agar. Plates were incubated at 36 °C for 20-24 hours. Susceptibility to tigecycline was defined based on clear zone diameters as susceptible (≥19 mm), intermediate (15-18 mm), or resistant (≤14 mm), based on the Food and Drug Administration (FDA) criteria for susceptibility zone diameters for *Enterobacteriaceae*. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria for *Escherichia coli*, isolates were categorized as resistant if the zone diameter was <15 mm and susceptible if the zone diameter was ≥18 mm[6].

Agar gradient test

Bacterial suspensions prepared to 0.5 McFarland turbidity units were inoculated onto MH agar (Oxoid, UK) and gradient antibiotic test strips (AB Biodisc, Sweden) were placed on the agar. Plates were incubated at 36 °C for 20-24 hours. The minimum inhibitory concentration (MIC) values reported by FDA and EUCAST in *Enterobacteriaceae* species were used to determine the susceptibility of tigecycline.

Broth microdilution test

MH bouillon (Oxoid, UK) was prepared according to the Clinical and Laboratory Standards Institute (CLSI) recommendations and added to 96-well microdilution plates. Tigecycline active ingredients were resuspended as *per* manufacturer's recommendations, stock solutions were prepared, and serial tigecycline dilutions (32-0.062 µg/mL) were prepared from stock. *A. baumannii* isolates were diluted to 0.5 McFarland turbidity units and further diluted to a ratio of 1:100. Bacterial suspensions were then added to microplate wells, except for those designated as negative controls, and incubated at 36 °C for 20-24 hours. The lowest antimicrobial drug concentration without bacterial growth was identified as the minimal inhibitory concentration required to inhibit the growth of organisms (MIC). According to the FDA criteria for *Enterobacteriaceae,* isolates were categorized as resistant if MIC was ≥8 µg/mL, intermediate if MIC was 4-6 µg/mL, and susceptible if MIC was ≤2 µg/mL. According to the EUCAST criteria for *Enterobacteriaceae,* isolates were categorized as resistant if MIC was >2 µg/mL and susceptible if MIC was ≤1 µg/mL[6].

Statistical analysis

Kruskal-Wallis test was used to determine the change in tigecycline resistance over years. Very major errors (VME) were considered in cases where BMT indicated resistance and the comparative method indicated susceptibility; major errors (ME) when an isolate was categorized as susceptible by BMT and resistant by the comparative method; and minor errors (mE) when an isolate was categorized as susceptible or resistant by BMT and intermediate by the comparative method, or *vice versa*. Categorical agreement (CA) was defined as the percentage of isolates recorded in the same susceptibility category by DDT, AGT and BMT.

Results

This study analyzed 94 multidrug-resistant *A. baumannii* isolates obtained from samples sent to our lab-

Table 1. BMT, DDT and AGT results, n (%)

According to the FDA criteria							
Method	Susceptible	Intermediate	Resistant				
BMT	89 (95)	4 (4)	1 (1)	VME*	ME	mE	CA
DDT	5 (5)	65(69)	24 (26)	0	23 (26)	63 (67)	8 (9)
AGT	94 (100)	0 (0)	0 (0)	1	0 (0)	4 (4)	89 (95)
According to the EUCAST criteria							
Method	Susceptible	Intermediate	Resistant				
BMT	88 (94)	5 (5)	1 (1)	VME	ME	mE	CA
DDT	16 (17)	54 (57)	24 (26)	0	24 (27)	51 (54)	19 (20)
AGT	93 (99)	1 (1)	0	1	0 (0)	5 (5)	88 (94)

DDT = disc diffusion test; AGT = agar gradient test; BMT = broth microdilution test; FDA = Food and Drug Administration; EUCAST = European Committee on Antimicrobial Susceptibility Testing; VME = very major errors; ME = major errors; mE = minor errors; CA = categorical agreement; *percentage of VME was not calculated because only one isolate was determined as resistant by BMT

oratory between January 2013 and December 2015. Out of 94 isolates, 25 (27%) were from samples obtained in 2013, 32 (34%) from samples collected in 2014, and 37 (39%) from samples obtained in 2015. Isolates were obtained from the following sources: deep tracheal aspiration (DTA, n=33; 35%), blood (n=31; 33%), wounds (n=17; 18%), sputum (n=10; 11%), and bronchoalveolar lavage (BAL, n=3; 3%).

Results according to the FDA criteria

Broth microdilution test showed 89 (95%) isolates to be susceptible to tigecycline, 4 (4%) were intermediate, and 1 (1%) was resistant. DDT showed that 5 (5%) isolates were susceptible to tigecycline, 65 (69%) were intermediate, and 24 (26%) were resistant. In contrast, AGT revealed that all isolates were susceptible to tigecycline.

Results according to the EUCAST criteria

Broth microdilution test showed that 88 (94%) isolates were susceptible to tigecycline, 5 (5%) were intermediate, and 1 (1%) was resistant. DDT showed 16 (17%) isolates to be susceptible to tigecycline, 54 (57%) were intermediate, and 24 (26%) were resistant. AGT showed that 93 (99%) isolates were susceptible and 1 (1%) was resistant to tigecycline. BMT, DDT and AGT results are shown in Table 1.

There were no significant differences in tigecycline resistance among samples obtained in 2013, 2014 and 2015, as evaluated by BMT. The only isolate found to be resistant had been obtained from a blood sample from 2015 (p=0.824). The distributions of MIC_{50} *versus* MIC_{90} values of tigecycline according to years are shown in Table 2.

Table 2. Distribution of MIC_{50} vs. MIC_{90} values of tigecycline according to years

Year	MIC_{50}	MIC_{90}
2013	0.5	2
2014	0.5	1
2015	0.5	2

MIC_{50} = minimal inhibitory concentration required to inhibit the growth of 50% of organisms in isolates; MIC_{90} = minimal inhibitory concentration required to inhibit the growth of 90% of organisms in isolates

Discussion

Tigecycline binds to the 30S ribosomal subunit and inhibits bacterial protein synthesis and is effective against many gram-positive and gram-negative bacteria[7,8]. Many resistant *A. baumannii* isolates have been detected in many facilities, especially in intensive care units, and treatment of such infections is complicated. Accurate detection of tigecycline susceptibility is important because it has been reported that *A. baumannii* infections respond faster to treatment with

tigecycline[9,10]. Jones *et al*. have reported results of their five-center study which assessed susceptibility of multidrug-resistant *A. baumannii* isolates. They found that a ≥19 mm zone diameter, as recommended by the FDA, led to an unacceptable error rate of 23%. However, assessment based on a zone diameter of ≥16 mm/≤12 mm resulted in an acceptable error rate of 9.7%[11]. Thamlikitkul *et al*. found that only 44.6% of *Acinetobacter* spp. isolates could be defined as susceptible to tigecycline when zone size was set at ≥19 mm. Further, they found that 96.6% of *Acinetobacter* spp. isolates could be categorized as susceptible if a diameter of ≥13 mm was used. They also report that susceptibility by DDT was as high as 99% when the zone diameter was ≥13 mm, and that DDT specificity was 100% when compared to the results obtained by BMT[12]. Gulhan *et al*. found that 3% of the isolates were resistant, 49% were intermediate, and 48% were susceptible when the zone diameter conditions were set at ≥19 mm and ≤14 mm. These percentages decreased to 1%, 1% and 97%, respectively, when the zone diameters were set at ≥16 mm and ≤12 mm[4]. Mansur *et al*. were able to detect 83% (25/30) susceptibility using DDT when the zone diameter for susceptibility was set at ≥16 mm[8]. According to the FDA and EUCAST, we recorded ME, mE and CA rates of 26%, 67%, 9% and 27%, 54%, 20% with DDT, respectively. These results imply that the MIC values, as determined by the FDA and EUCAT, are not consistent with the zone diameter results. Therefore, when determining tigecycline susceptibility, the results of BMT may be more precise if the isolates are initially categorized as resistant or intermediate by DDT. Furthermore, given the ease of using DDT, it is essential that studies on tigecycline susceptibility precisely define zone diameter for the three categories of resistant, intermediate and susceptible isolates.

Studies using AGT demonstrate different results. Navon-Venezia *et al*. found 66% of multidrug-resistant *A. baumannii* isolates to be resistant, 12% intermediate and 22% susceptible to tigecycline when AGT was used[13].Thamlikitkul *et al*. report on 25% resistance to tigecycline in *A. baumannii* isolates, as determined by AGT, to be inaccurate[12]. Similarly, Mansur *et al*. also state that 30% resistance to tigecycline in *A. baumannii* isolates determined by AGT method was inaccurate[8]. Bedenic *et al*. have reported that AGT (E-test) did not provide reliable results[14]. Interestingly, Akın *et al*. report rates of tigecycline resistance to be 5.3% by BMT and 17.9% by AGT[1]. Bogaerts *et al*. did not detect tigecycline resistance by AGT in carbapenem resistant *A. baumannii* isolates, and Nayman-Alpat *et al*. did not detect resistance to tigecycline by AGT in 100 *A. baumannii* isolates from various clinical samples[15,16]. Contrary to this, we recorded ME, mE and CA rates of 0%, 4%, 95% and 0%, 5%, 94% with AGT, according to the FDA and EUCAST criteria, respectively. In agreement with our observations, Zarete *et al*. have also reported that the results obtained by AGT and BMT methods are similar and that AGT is suitable for detecting tigecycline resistance[17]. Fernandez-Mazarrasa *et al*. found that resistance rates determined by AGT were different if MH agar from different manufacturers was used and that higher levels of tigecycline resistance were associated with the concentration of manganese in the medium[18]. Therefore, it is possible that variations in the sensitivity profiles reported may be due to differences in the concentration of medium components such as manganese.

With respect to change in resistance over time, in their study conducted in the United States, Loan *et al*. have reported a susceptibility rate of 91.5% for tigecycline in *A. baumannii* isolates[19]. Sohail *et al*. in their study from Pakistan conducted between 2012 and 2014 found this rate to be 99.3%[20]. Sader *et al*. in a study conducted in 11 centers in Latin America between 2011 and 2014 have reported a MIC_{50} value of 1 and MIC_{90} value of 2 for tigecycline in *A. baumannii* isolates[21]. Similarly, studies from Turkey report high tigecycline susceptibility rates. Specifically, Akın *et al*. found a tigecycline susceptibility rate of 94.7% in *A. baumannii* isolates between 2006 and 2008, while in the study by Mansur *et al*. it was 100% and in the study by Direkel *et al*. it was 96.2%[1,8,22]. We showed a tigecycline susceptibility rate of 95%, which is comparable with those reported in the literature. Collectively, these observations suggest that because the susceptibility of *A. baumannii* isolates to tigecycline has not significantly changed over years, tigecycline might still be an appropriate treatment option for infections caused by multidrug-resistant *A. baumannii*. However, as there might be regional differences in susceptibility due to antibiotic usage policies, it would be essential to precisely determine tigecycline susceptibility and administer treatment accordingly.

Conclusions

In conclusion, the AGT is a safe method for determining susceptibility when the appropriate MH agar

is used, as it can be easily performed. The incompatibility between the results of DDT and BMT methods necessitates further confirmation of resistance or intermediate susceptibility of isolates using BMT.

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Sažetak

ISPITIVANJE OSJETLJIVOSTI NA TIGECIKLIN MULTIREZISTENTNIH IZOLATA *ACINETOBACTER BAUMANNII* TESTOVIMA DISK DIFUZIJE, AGAR GRADIJENTA I MIKRODILUCIJE U BUJONU

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Primjena tigeciklina postaje sve važnija zbog visoke razine otpornosti na antibiotike u izolatima *Acinetobacter baumannii* (*A. baumannii*). U ovoj prospektivnoj studiji dobiveni su multirezistentni izolati *A. baumannii* iz različitih uzoraka tkiva i tekućine bolesnika primljenih ili liječenih na različitim klinikama, koji su testirani u Laboratoriju za mikrobiologiju Medicinskog fakulteta Sveučilišta Duzce u razdoblju od siječnja 2013. do prosinca 2015. godine. Otpornost na tigeciklin u multirezistentnim izolatima *A. baumannii* analizirana je primjenom testa disk difuzije (*disc diffusion test*, DDT), testa gradijenta agara (*agar gradient test*, AGT) i zlatnog standardnog testa (*broth microdilution test*, test mikrodilucije u bujonu, BMT). U studiji su ispitana 94 izolata *A. baumannii* rezistentna na više lijekova. Metodom BMT utvrđeno je da su 89 (95%), 4 (4%) izolata i 1 (1%) izolat *A. baumannii* osjetljivi, srednje osjetljivi i rezistentni na tigeciklin. Primjenom kriterija FDA, stope velike greške (ME), male greške (mE) i kategoričkog poklapanja (CA) za DDT iznosile su 26%, 67% odnosno 9%. Nasuprot tome, stope ME, mE i CA za AGT bile su 0%, 4% odnosno 95%. Otpornost na tigeciklin procijenjena metodom BMT nije pokazala povećanje između 2013. i 2015. godine. Izolate za koje je metodom DDT utvrđeno da su otporni ili srednje otporni treba potvrditi metodom BMT. Zahvaljujući jednostavnoj primjeni AGT je sigurna metoda otkrivanja osjetljivosti.

Ključne riječi: *Acinetobacter baumannii; Test gradijenta agara; Test mikrodilucije u bujonu; Test disk difuzije; Multirezistentnost; Tigeciklin*