ORIGINAL ARTICLE − OPEN ACCESS

In Vitro Susceptibility Testing of Rifampin Against *Acinetobacter Baumannii*: Comparison of Disk Diffusion, Agar Dilution, and E-test

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ABSTRACT

Objective: Resistance against a wide variety of antibiotics is one of the prominent characteristics of *Acinetobacter baumannii*. The purpose of this study was to investigate the *in vitro* susceptibility testing of *A. baumannii* isolates to rifampin and the examination of the value of routine antibiogram with disk diffusion, E-test, and agar dilution methods on collected isolates from a tertiary hospital in north-west Iran.

Materials and Methods: Susceptibility of 68 clinically isolated *A. baumannii* against rifampin using three *in vitro* methods was investigated. For the E-test method, the Pachon–Ibanez's and Saballs's study criteria were used. The Pachon–Ibamez criteria were used for agar dilution method. For disk diffusion, the standard Kirby–Bauer diffusion method was used. The area under curve (AUC) was used to determine the appropriate methods. The methods were interpreted using sensitivity, specificity, and negative and positive predictive values.

Results: *A. baumannii* susceptibility to the rifampin according to the E-test was 41.2% (Panchon–Ibanez criteria) and 32.4% (Saballs's criteria). The susceptibility was 29.4% according to the agar dilution method for the Panchon–Ibanez criteria, 2.9% according to the agar dilution method for the Saballs's criteria and, and 1.5% according to the disk diffusion methods. The results of the E-test method according to Pachon–Ibanez's and Saballs's criteria in comparison with the result of the agar dilution method according to Pachon–Ibanez's criteria had the highest AUC.

Conclusion: According to the susceptibility testing of rifampin against *A. baumannii*, the E-test method has a higher diagnostic value than the agar dilution and disk diffusion methods.

Keywords: *Acinetobacter baumannii*, difampin, disk diffusion, antimicrobial tests, microbial sensitivity tests, antibiotic resistance

INTRODUCTION

Acinetobacter baumannii is a gram-negative, catalase-positive, glucose-non-fermentative, generally encapsulated, non-motile, and aerobic coccobacillus that grows on the numerous human sources and may be colonized on a healthy adult's skin and on the adult and infant pharynx. Long-term hospitalization, intensive care unit setting, mechanical ventilation, antibiotic therapy, recent surgery, invasive procedures, and underlying diseases are the risk factors for the colonization and infection of multiple-drug resistant (MDR) *A. baumannii* (1–3).

It is an opportunistic pathogen that causes nosocomial infections. The respiratory tract is the common site of infection. *A. baumannii* isolates are frequently resistant to multiple classes of antibiotics and treatment of the MDR strains is challenging in the health care setting. At least 45 genomic resistant *A. baumannii* species have been described by Fournier et al. (4) and MDR *A. baumannii* incidence is increasing worldwide (5).

Since many *A. baumannii* species are pan-resistant to antibiotics, the treatment options are limited. Thus, the management of *A. baumannii* infections has become a public health problem in many regions (6). Hence, studies about the resistance of *A. baumannii* isolates collected from different regions to various antibiotics are of great importance.

Rifampin, a semi-synthetic derivative of rifamycin, has *in vitro* activity against some gram-positive and gram-negative bacteria, mycobacteria, and chlamydia. Resistant mutants exist among all pathogens when rifampin is used in monotherapy conditions (7). Clinically, it is not only an important anti-tuberculosis drug, but is also used in combination with others in treatment of critical staphylococcal infections (8). Since the use of rifampin as monotherapy in the treatment of *A. baumannii* infections causes rapid drug resistance to this antibiotic, it is used in combination with other antibiotics (9).

To increase laboratory information is a clinical challenge with *A. baumannii*, especially MDR isolates. Therefore, the study of *in vitro* susceptibility of this organism against rifampin alone and also study of the possibility of trusting the routine methods for susceptibility tests including disk diffusion, E-test, and agar dilution, has been investigated.

MATERIALS and METHODS

After obtaining approval from the Institutional Review Board (IRB), 68 clinical isolates of A. baumannii obtained from 68 hospitalized patients in different wards of Sina Hospital, a tertiary care hospital in Tabriz, north-west Iran, were investigated. The collected isolates were from different clinical samples including blood, urine, wound, sputum, and chest tube drainage.

The standard classical microbiological testing methods used to identify A. baumannii isolates included assessing the morphology of the colony (non-pigmented, domed, mucoid, smooth, and pitted surfaces), oxidase test (negative), motility (non-motile), catalase test (positive), and citrate test (positive), due to the growth at 37°C and 47°C and acid production from glucose on the O/F medium. Also, the VITEK® 2 automatic microbial identification system (bioMérieux, Marcy l'Etoile, France) was used for recognition of gram-negative bacilli for originally identified strains of the A. baumannii-A. calcoaceticus complex. All isolates were confirmed by the polymerase chain reaction for the presence of blaOXA-51-like gene to differentiate between the types of A. baumannii (10).

Susceptibility against rifampin was assessed by disk diffusion, agar dilution, and E-test. In the disk diffusion method, the rifampin susceptibility of all isolates was assessed using a 5 µg rifampin disk (HiMedia, Tarnaka, India) and the standard Kirby–Bauer diffusion method. Cultured bacteria, after 24 hours of incubation, were used for suspension preparation at 0.5 McFarland. This solution was placed on surfaces with a 1.5–2 cm interval for 5 minutes. After 18–24 hours of incubation in 37°C, halo diameters of inhibition of bacterial growth were measured. The results, obtained according to the criterion used in the Thapa study (11), were divided into susceptible (zone diameter >20 mm) and not susceptible (zone diameter ≤20 mm).

In the agar dilution method, rifampin minimum inhibitory concentrations (MICs) were specified. First, a stock rifampin powder solution (CKD Bio, Seoul, Korea) was prepared according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (12) and the manufactures' guide. This solution was prepared in various dilutions, in the range of 0.125–512 µg/mL, using the following formula:

Powder weight=[Volume (mL)×Concentration (µg/mL)]/Potency (µg/mL)

The rifampicin solution was mixed with prepared Muller–Hinton agar and poured in plates on a flat surface with 4 mm depth. Plates containing Muller–Hinton agar and rifampin in different dilutions were labeled and kept in closed bags at a temperature of 2–8°C. For preparing bacterial suspension, colonies were poured directly onto the sterilized physiologic serum and the turbidity was made as standard at 0.5 McFarland, so that bacteria could be formed with 108 colony-forming units (CFU)/mL dilutions. This solution was diluted with a physiologic serum to 1/10 (107 CFU/mL).

The agar plates were divided into parts to estimate the inoculated points. 1–2 µL from 107 CFU/mL sample was placed on agar with a standard pipette, so that the final 104 CFU/spot dilutions were provided. After absorption of inoculated bacterial moisture to agar in room temperature, plates were incubated in 35–37°C for 10–20 hours.

Table 1. *Acinetobacter baumannii* sensitivity in disk diffusion (zone diameter), agar dilution (rifampin concentration), and E-test methods

	Mean±SD	Range
Zone diameter (mm)	14.47±3.88	6–22
Rifampin concentration (mg/L)	62.38±77.46	2–256
E-test strip (mg/L)	15.75±13.90	1–32

SD: Standard deviation

Finally, MIC was determined as the minimum rifampin dilution that inhibited the growth completely and considered rifampin susceptible when the growth was as a colony alone or seen as a light haze. The results were divided into two groups: susceptible (rifampin dilution ≤4 mg/l) and not susceptible (rifampin dilution >4 mg/l) according to criteria used in Pachon–Ibanez's study (13), and susceptible (rifampin dilution <4 mg/l) and not susceptible (rifampin dilution ≥4 mg/l) according to criteria used in Saballs' study (14).

The E-test method was prepared according to the manufacturer's instructions. A bacterial solution was placed in physiologic serum, set to the 0.5 McFarland standard, and cultured on Muller–Hinton agar (HiMedia, Tarnaka, India), after which the plates were placed at room temperature for 10 minutes to absorb extra moisture. The E-test strips (bioMérieux, Askim, Sweden) were placed at room temperature for 20 minutes before use. Then, they were placed carefully on an inoculated media surface and were finally incubated at 37°C in normal atmosphere for 18–24 hours. Thereafter, MIC was determined on the basis of the site that the formed ellipse crossed the stripe. The results were divided into two groups: susceptible (≤4 mg/l) and insusceptible (>4 mg/l), according to criteria used in the Pachon–Ibanez study (13), and susceptible (<4 mg/l) and insusceptible (≥4 mg/L) according to criteria used in the Saballs study (14).

The results were statistically analyzed using descriptive statistical methods (frequency, percentage, and mean±SD) and calculation of sensitivity, specificity, and positive and negative predictive values using the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, IL, USA). In this study, the receiver operating characteristic curve analysis was considered for calculation of the area under curve (AUC) with a 95% confidence interval (CI) for comparison between the methods.

RESULTS

The values of susceptibility testing of rifampin against A. baumannii isolates compared with three studied methods (disk diffusion, E-test, and agar dilution) are shown in Table 1. The numbers and percentage of susceptibility isolates with three methods are presented in Table 2. According to the results, A. baumannii had the highest resistance to rifampin in the disk diffusion and agar dilution (Saballs criteria) methods and highest sensitivity to rifampin in the E-test (in both Panchon–Ibanez criteria and Saballs criteria methods). Also, the results of the statistical analysis for the comparison of sensitivity, specificity, positive & negative predictive values, and accuracy of the methods are summarized in Table 3. According to these results, the E-test (Panchon–Ibanez criteria and Saballs

Table 2. *Acinetobacter baumannii* rifampin sensitivity values in disk diffusion, agar dilution, and E-test methods

	Disc diffusion		Agar dilution (Panchon-Ibanez Criteria)		Agar dilution (Saballs Criteria)		E-test (Panchon-Ibanez Criteria)		E-test (Saballs Criteria)	
	n	%	n	%	n	%	n	%	n	%
Sensitive	1	1.5	20	29.4	2	2.9	28	41.2	22	32.4
Resistant	67	98.5	48	70.6	66	97.1	40	58.8	46	67.6

Table 3. Diagnostic accuracy in disk diffusion, agar dilution, and E-test methods

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	95% CI
DD in comparison to AD (Panchon-Ibanez Criteria)	5	100	100	71	0.563	0.489–0.618
DD in comparison to AD (Saballs Criteria)	0	98	0	97	0.618	0.537–0.672
DD in comparison to Etest (Panchon-Ibanez Criteria)	3	100	100	59	0.403	0.374–0.499
DD in comparison to Etest (Saballs Criteria)	4	100	100	68	0.436	0.381–0.512
E-test (Panchon-Ibanez Criteria) in comparison to AD (Panchon-Ibanez Criteria)	95	81	67	97	0.927	0.871–0.898
E-test (Panchon-Ibanez Criteria) in comparison to AD (Saballs Criteria)	100	60	7	100	0.721	0.703–0.798
E-test (Saballs Criteria) in comparison to AD (Panchon-Ibanez Criteria)	80	87	72	91	0.817	0.793–0.894
E-test (Saballs Criteria) in comparison to AD (Saballs Criteria)	100	69	9	100	0.742	0.653–0.771
E-test (Panchon-Ibanez Criteria) in comparison to AD (Saballs Criteria)	78	100	100	86	0.791	0.703–0.834
AD (Panchon-Ibanez Criteria) in comparison to AD (Saballs Criteria)	100	72	10	100	0.687	0.561–0.713

DD: Disk diffusion; AD: Agar dilution; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under curve; CI: Confidence interval

criteria) method, in comparison to agar dilution, (Panchon–Ibanez criteria) has the highest AUC among all the methods.

DISCUSSION

A. baumannii is a gram-negative coccobacillus that causes nosocomial and sometimes severe community-acquired infections (15). Immunosuppressed and critically ill patients, particularly burn patients, are at a high risk of acquiring *A. baumannii* infections. Multiple aspects can have an effect on the propensity of *A. baumannii* to cause clonal outbreaks, including those that are ubiquitous in nature (soil, water, animals, and humans), hospital conditions (furniture, equipment, bed rails, sinks, and air vents), the orangism's capability to survive for prolonged period of time in hospital conditions, and the relative ease with which it acquires resistance against multiple antibiotics (16–19).

There are some clinical reports about the successful use of rifampin for the treatment of critical *A. baumannii* infections. Bassetti et al. (20) reported that critically ill patients with pneumonia or bacteremia were successfully treated with colistin plus rifampicin. Clinical and microbiologic responses were observed in 76% of the patients and the overall infection-related mortality was 21%. In an *in vitro* study, Wang et al. (21) revealed that imipenem and rifampicin showed potential synergistic antimicrobial activity against *A. baumannii*.

Gleeson et al. (22) reported the case of a 34-year-old female, which involved *A. baumannii* nosocomial meningitis after neurosurgery. On day 13, she remained febrile and her cerebrospinal fluid (CSF)

culture demonstrated *A. baumannii*, despite receiving treatment with various antibiotics, including meropenem, vancomycin, gentamicin, and metronidazole, until day 13, when gentamicin and metronidazole were discontinued and intravenous rifampin was added to the meropenem. About 16 days after the treatment, the patient became afebrile and her CSF analysis demonstrated an improvement in the negative cultures, whereas rifampin susceptibility testing by disk diffusion revealed a 14 mm zone of inhibition.

In vitro susceptibility testing often provides a guideline for the selection of an appropriate antibiotic. A number of various laboratory methods such as disk diffusion, agar dilution, E-test, and molecular techniques have been used to assess the activities of antibiotics against bacteria. Low cost and simplicity are two important properties of the disk diffusion method. The agar dilution method has accurate results but is technically troublesome. E-test as a variation of the Kirby–Bauer disk diffusion has been validated for many organisms as compared to the broth agar dilution method and it has shown excellent correlation between E-test and dilution test (4, 23).

Several studies have been done to compare the diagnostic value of different laboratory methods. Baker et al. (24) studied the susceptibility of 140 gram-negative and 55 gram-positive isolates against various antibiotics. The E-test yielded excellent agreement results when compared with the disk diffusion and agar dilution tests.

Heijdan et al. (25) studied the susceptibility of 109 carbapenems resistant Pseudomonas aeruginosa against polymyxin B and colistin. Results showed that the accuracy of disk diffusion assay was unsatisfactory because the polymyxins diffused poorly into the agar. In a study by Swenson et al. (26), the susceptibility of 196

Acinetobacter spp. isolates against 19 antibiotics were evaluated with the broth microdilution and disk diffusion methods. The results of the broth microdilution and disk diffusion were concordant for most non-β lactam agents but not for Tetracycline. Gulmez et al. (27) evaluated the susceptibility of 25 Stenotrophomonas maltophilia isolates against some antibiotics. Agar dilution was considered as the reference method. The results showed that the disk diffusion method for colistin examination provided inaccurate and unreliable results and the ciprofloxacin and ticarcillin/clavulanate examination was a poor agreement between the disk diffusion and the reference methods. It seems that, in spite of the wide use of disk diffusion in laboratories and the clinical use of its results, the diagnostic value of this procedure is less, especially for some antibiotics such as colistin.

CONCLUSIONS

In the current study, the statistical comparison of three methods, i.e., disk diffusion, agar dilution, and E-test for the evaluation of the susceptibility of rifampin against *A. baumannii* demonstrated a higher AUC between E-test (in both Saballs and Pachon–Ibanez criteria) and agar dilution methods with criteria that were used in the Pachon–Ibanez study. Disc diffusion was an inaccurate and unreliable method for testing the susceptibility of *A. baumannii* against rifampin. This may be relevant to the poor diffusion of rifampin into agar, differences in the defined breakpoint, or the quality of the used disk.

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