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Comparison of Disk Diffusion and E-Test Methods to Determine Antimicrobial Activity of Ceftazidime and Ciprofloxacin on Clinical Isolates of *Acinetobacter baumannii*

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ABSTRACT

This study is aimed to find a specific method to test the clinical isolates of Acintobacter Baumanniiin terms of antimicrobial susceptibility and resistance to two drugs of Ceftazidime and Ciprofloxacin using E-test and Disk Diffusion methods. Totally 100 samples were collected from hospitalized patients at the general hospitals of Kerman Province, Southeastern, Iran between November 2013 to April 2014. They were identified by standard microbiological methods. Susceptibility by Disk diffusion and MIC by E-test were performed according to the Clinical and Laboratory Standards Institute breakpoints. In the disk diffusion method for ciprofloxacin antibiotic, the Acinetobacter specimens were reported to be 85% resistant, 4% intermediate, and only 11% susceptible. Also, for Ceftazidime antibiotic, the specimens were reported as 75% resistant, 15% intermediate, and only 10% susceptible. In the E-test method for ciprofloxacin antibiotic, the Acinetobacter specimens were reported to be 95% resistant, 0% intermediate, and 5% susceptible. Also, for Ceftazidime, the specimens were reported to be 93% resistant, 4% intermediate, and 3% susceptible. After performing the statistical Chi square test at confidence level of 95%, the P value for these two antibiotics was obtained ($p<0.199$) in both methods. The findings of present study revealed the high resistance of this bacterium in Kerman Province and feeling the necessity of thinking of some strategies and solutions for reducing that microbial resistance as well as paying more attention to the selective treatments, antibiotic treatment course duration, and other instances that should be taken into account in any antibiotic diet in order to prevent and avoid such high levels of microbial resistance in our country.

Key words: *Acintobacter*; antibiotic; resistance; disk diffusion; T-test

INTRODUCTION

Acinetobacter is a genus of gram-negative and strictly aerobic bacteria naturally found on skin and in throat or intestine. *Acinetobacter baumannii* is responsible for nosocomial infections, especially in Intensive Care Units (ICU) and Burn Therapy Units (BTU) [1]. *A. baumannii* is also responsible for nosocomial pneumonia, wound infections, bacteremia, urinary tract infection, meningitis, endocarditis, osteomyelitis and keratitis [1,2]. Developing simultaneous resistance against various antibiotics is a property of this microorganism. Antibiotics including carbapenems and the third generation cephalosporins are the most important risk factors causing the resistance of this microbe [3]. Among the mechanisms which create resistance we can name hydrolysis by beta-lactamase secreted from the protein membrane of the outer surface of this microorganism as well as the penicillin-bound proteins [3, 4]. Today, one of the problems of fighting against *Asinobacter spp.* in hospitals and clinics has been reported to be the resistance of this microorganism to carbapenems, aminoglycosides, and fluoroquinolones [5].

Since existence of this microorganism is one of the causes of infection in the Infectious Diseases Ward, ICU, and other hospital wards, the injective Ciprofloxacin and ceftazidime antibiotics are prescribed for its treatment. As

microbial resistance has been always one of the main problems in consumption of the antibiotics, controlling this resistance requires precision in their consumption.

One of the methods which help preventing microbial resistance is assessment of antimicrobial activity of antibiotics. It is recommended to investigate the antimicrobial activity of the antibiotics during their consumption, especially in the hospitals where there are more resistant microbes. To do this, there are various methods including MIC, E-test, and Disk Diffusion test by which the specific antibiotic of each microorganism is identified and then its minimum effective concentration for the microorganism is specified [6].

This study is aimed to find a specific method to test the clinical isolates of *A. baumannii* in terms of antimicrobial susceptibility and resistance to two drugs of Ceftazidime and Ciprofloxacin using E-test and Disk Diffusion methods. Ciprofloxacinis one of the carbapenem antibiotics with beta-lactam ring widely used against the gram negative microorganisms such as *Acinetobacter* which is found in the abdominal, bone, ulcers, urinary tract, skin, and other infections. Ceftazidime belongs to the class of the third generation Cephalosporins and is widely and specifically used for treatment of the gram negative *Acintobacter* infections. Both of these drugs are used through injection and intravenous infusion [5].

MATERIALS AND METHODS

Bacterial isolates

Totally 100 samples were collected from hospitalized patients at the general hospitals of Kerman Province, Southeastern, Iran between November 2013 to April 2014. The isolates were collected from different specimens, including blood, cerebrospinal fluid (CSF), tracheal secretions, wound, and urine. All isolates were routinely cultured on the blood agar and Macconkey media, and then the plates were incubated at 37° C for 24-48h. In case of observing the growth after staining and observing gram negative cocci and diplococci, the specimens were examined using oxidase test. In the next step, the negative oxidase specimens were tested and definitely identified using biochemical tests such as motility tests, citrate test, culturing on the glucose-containing medium, and growth at temperature of 42-44 °C.

Preparation of microbial inoculation for disk diffusion method

Each series of microbe culturing in the plates requires a 24-hour fresh culture. So,24 hours earlier, another culture is prepared from the reserve (stored) culture in order to use anewand fresh 24h culture of the microorganisms. First, a colony of the culture containing microorganism was solved in normal saline (9.0 %) and, then, some of this suspension was poured by a sterile pipette into the normal saline-containing sterile capped pipes so that the opacity of about 0.5 McFarland, which was already prepared, was achieved. The resulting suspension contained about 1.5×10^8 microorganisms/ml.

Microbial suspension transfer in disk diffusion method

In sterile conditions, 50 μl of the standardized microbial suspension (7.5×10^6 microorganisms) was taken by a sampler and poured into the test tubes containing 1ml of the sterile normal saline. The final microbial concentration (7.5×10^4 microorganisms) was taken and point-cultured on the Mullerhinton agar culture medium without heavy metals or antibiotics. In each of the dilutions, point-culturing was performed separately and similarly. Then, the prepared plates were put in the incubators with temperature of 37°C and, after 24 h, examined for growth or lack of growth. The control plates were also incubated for 24h at 37°C and then examined for their growth or lack of growth.

Preparation of 0.5 McFarland standards

This solution was used to standardize the microbial leachate solution for performing the resistance test. The 0.5 McFarland standard solution is obtained from combining barium chloride and sulfuric acid. It creates an opacity close to the opacity resulted from $10^8 \times 1.5$ micro-organisms in 1ml of inoculation. To prepare this solution, as it is formulated, 0.05ml of barium chloride (with aforementioned properties) was mixed with 9.95ml of 1% sulfuric acid and then stored in screw capped glass tubes. The 0.5McFarland standard remains stable for 6 months in the refrigerator under such conditions.

Disk diffusion test

Among the microbial susceptibility determination methods, the Disk Diffusion test is the most common one. In this method, after isolation of bacterium, some of the bacterial colony is dissolved in a physiology serum to reach 0.5 McFarland; then, it is transferred to the Mullerhinton agar culture medium. Afterwards, the relevant antibiogram disks are put in the culture medium by distance of 12mm from each other. After capping them, they are incubated at 36°C for 24h. Next, under the light, the diameter of inhibition halo (growth inhibition halo) is examined and

measured by ruler and compared with the reference tables, and then reports are provided as susceptible, resistant, or intermediate. Measurement of the inhibition halo must be done always with ruler. The length of the ruler should pass through the disk center to measure the diameter; all of these actions should be performed under the light. The susceptibility determination standard based on the diameter of the inhibition halo for was shown in Table 1 [7].

Table 1. Standard diameter of inhibition halo for Ciprofloxacin and Ceftazidine in disk diffusion method

Antibiotic	Susceptible	Intermediate	Resistant
Ceftazidine	≥ 17 mm	14-16 mm	≤ 13 mm
Ciprofloxacin	≥ 21 mm	16-20 mm	≤ 15mm

Determination of microbial susceptibility by E-test (Epsilometr Test)

This method is similar to disk diffusion method except that, in this method, special plastic tapes are used instead of antibiogram discs and the antibiotics are distributed as concentration gradient [6-8]. Further, in this method, MIC is obtained from the intersection of the inhibition halo in the culture medium. These antibiotic tapes were provided by Swedish Company of AB BIODISK according to whose catalogs the terms "susceptible", "intermediate", and "resistant" were defined (Table 2) [7].

Table 2. Standard MICs for Ciprofloxacin and Ceftazidine in T-test method

Antibiotic	Susceptible	Intermediate	Resistant
Ceftazidine	≤ 8 µg/ml	16 µg/ml	32 µg/ml ≥
Ciprofloxacin	≤ 1 µg/ml	2 µg/ml	4 µg/ml ≥

Statistical analysis

To analyze the obtained data, SPSS Software Version-16 was used. Data was presented in two separate tables, each introducing one of the used methods. For each table, three terms of susceptible, intermediate, and resistant were defined in three columns; then, the Chi square test was used for data analysis. To test validity of the disk diffusion method, results of E-test were considered as the golden standard and, then, susceptibility, specificity, and positive and negative predictive values of this test were calculated and reported at 95% confidence level. The value of $p<0.05$ was assumed as the significant statistical difference.

RESULTS

Collected samples

In this study, 100 specimens were collected from 100 different patients, to whom the definition of hospital infection applied, and entered into the research after confirmation of the infectious diseases specialist of the hospital and central lab with the following conditions: (i) Their disease should be caused at least 48-72h after the patient´s admission to the hospital, {ii) At the time of admission, the person should have no obvious symptoms of the relevant infection, (iii) The individual should have the relevant criteria of a specific infection for defining the hospital infection. That is, the biochemical patterns of all the 100 isolated specimens identified as *A. baumannii* should be the same (Table 3).

Table 3. Frequency of collected specimens

Specimen type	Number (%)
Trachea	38
Phlegm	24
Urine	21
Blood	5
Catheter	5
Wound culture	3
Ascites fluid	2
Cerebrospinal fluid	2
Total	100

The collected specimens comprised of 67% males and 33% females with average age of 44±28.2 years old, and 18 subjects were above 60 years old. The most common infections caused by this bacterium were pneumonia (62%), UTI (21%), primary blood septicemia (10%), abdominal infections and meningitis (4%), and wound infection (3%).

Disk diffusion

In the disk diffusion method, the diameter of the inhibition halo was compared with the NCCLS reference (National Committee for Clinical Laboratory Standard) and the catalogues of the manufacturer company (Table 4). In the disk diffusion method for ciprofloxacin antibiotic, the *Acinetobacter* specimens were reported to be 85% resistant, 4% intermediate, and only 11% susceptible. Also, for Ceftazidime antibiotic, the specimens were reported as75% resistant, 15% intermediate, and only 10% susceptible.

Table 4. Results of disk diffusion method

Antibiotic	Susceptible	Intermediate	Resistant
Ciprofloxacin	11%	4%	85%
Ceftazidine	10%	15%	75%

E- Test method

In the E-test method, the MIC report was based on the report of the inhibition halo's intersection with the E-test tape. The obtained values were compared with the catalogue sent by the Swedish manufacturer company. The following results were obtained (Table5).In the E-test method for ciprofloxacin antibiotic, the *Acinetobacter* specimens were reported to be 95% resistant, 0% intermediate, and 5% susceptible. Also, for Ceftazidime, the specimens were reported to be 93% resistant, 4% intermediate, and 3% susceptible.

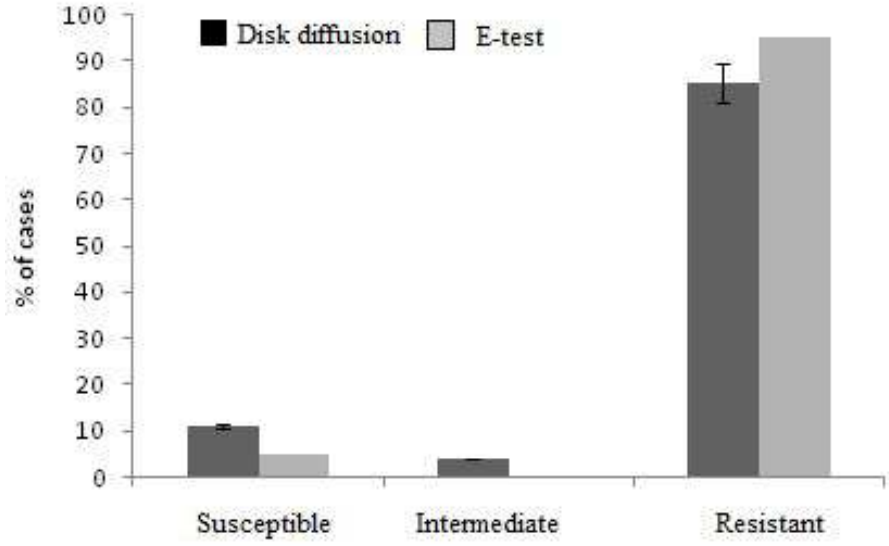

Figure 1- Comparison of results obtained for Ciprofloxacin in two methods

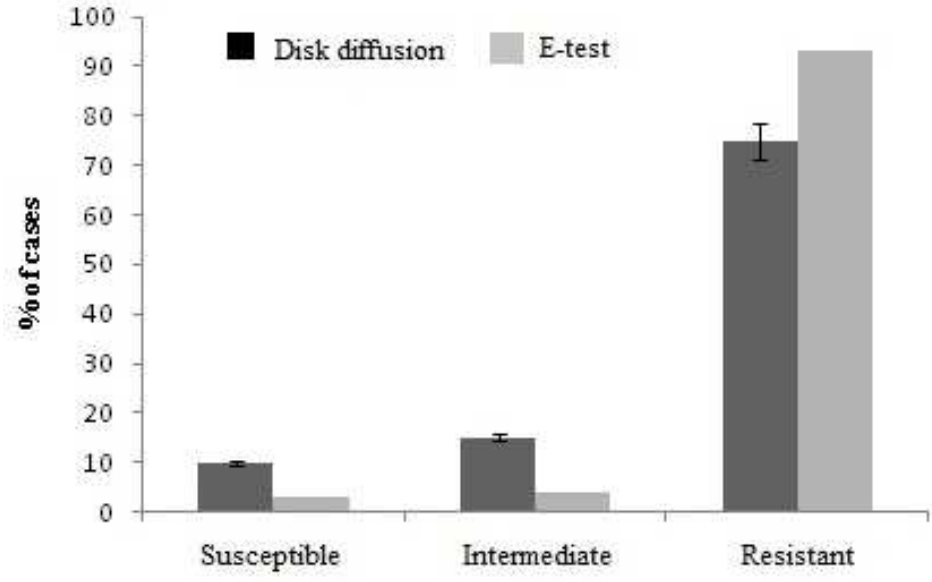

Figure 2- Comparison of results obtained for Ceftazidime in two methods

Table 5. Results of E-test method

Antibiotic	Susceptible	Intermediate	Resistant
Ciprofloxacin	5%	0%	95%
Ceftazidine	3%	4%	93%

Comparison of results obtained for Ciprofloxacin in two methods

Figures 1 and 2 show the comparison of results obtained for Ciprofloxacin in two methods of disk diffusion and T-test. After performing the statistical Chi square test at confidence level of 95%, the P value for these two antibiotics was obtained ($p<0.199$) in both methods.

DISCUSSION

In the recent century, due to the growing number of the patients suffering from immune deficiency, malignancy, chronic diseases, AIDS, and expansion of the ICUs, incidence of the hospital infections is one of the major problems in health and medical centers [10, 11]; while, as a consequence of the improper use of antibiotics, incompletion of the therapeutic course, and over-prescription of the antibiotics have caused the microbes, previously treated by their specific antibiotics, to acquire resistance against these antibiotics through different mechanisms [12]. Perhaps, in this regard, identification of this resistance and finding a selective treatment for them is considered as one of the most important challenges ahead. In any case, the first step to overcome this issue is answering the question that how much resistant is the given microbe to the antibiotic treatments. There are methods, with specific conditions, whose application differs depending on different requirements of a hygienic unit such as ICU has [13]. This study is aimed to assess the validity of the old disk diffusion method compared to the more expensive and, of course, more susceptible method of E-test against the negative gram bacteria such as *A. baumannii* which causes some prevalent hospital infections and is often treated by Ciprofloxacin and Ceftazidine and other antibiotics of the same family. 100 specimens, with proved *Acinetobacter*, were isolated from the ICU of General Hospitals of Kerman Province, Iran between November 2013 to April 2014.

The collected specimens included tracheal secretion (38%), phlegm (24%), urine (21%), blood and catheter (5%), wound culture (3%), ascites fluid (2%), and spinal fluid (2%). Regarding the sites of specimen collection, it can be said that 62% of the cases were related to the pneumonia caused by *Acinetobacter*, 21% to the urinary tract infections, 10% to the initial blood septicemia, 4% to the abdominal infections and meningitis, and 3% to the wound culture medium that can be considered as the most common site of the involvement of this bacteria for the patients in the ICU of the hospital. Based on the results of both methods, the microbial susceptibility of Ciprofloxacin in disk diffusion and E-test methods was reported to be 85% resistant 95% resistant, respectively, and 90% resistant on average; while, previous studies conducted in Oman using the disk diffusion method had reported the microbial resistance to be 50% in Oman [14], 33.8% in Turkey [15], and 40% in America [16] which indicates the higher prevalence of the microbial resistance of this microbe against Ciprofloxacin antibiotic in Iran.

Results obtained for Ceftazidime approved that the microbial resistance is very high in Iran so that, for this antibiotic, the microbial resistance was reported to be 75% and 93% in the disk diffusion method and in E- test method, respectively, and 90% on average. While a similar study in Oman showed that the microbial resistance of the *A.baumannii* specimens in the hospital was 50% in Oman [14], 57.8% in Turkey [15], 5.8% in Japan [17], and 40% in America [16].

Comparison of the bacterial resistance in two methods for Ciprofloxacin and Ceftazidime antibiotics led to the following data: in disk diffusion method for Ciprofloxacin, 11%, 4%, and 85% of the specimens were susceptible, intermediate, resistant, respectively; however, in E-test, 5% of the specimens were susceptible, 0% were intermediate, and 95% were resistant.

For Ceftazidime, in disk diffusion method, 10%, 15%, and 75% of the specimens were susceptible, intermediate, resistant, respectively; while, in E-test, 2% of them were susceptible, 4% were intermediate, and 93% were resistant. By statistical comparison using SPSS software and Chi Square test, the P-value of 0.199 was obtained; therefore, regarding the zero hypothesis, stating the lack of a significant relationship between these two methods of microbial susceptibility determination at ($p<0.05$), we had to reject the zero hypothesis and accept that the disk diffusion method is still a suitable test for susceptibility determination for this bacterium.

In a similar study conducted in Sina Hospital in Tehran, comparison of these two methods and the observed resistance of the tested bacteria led to the conclusion that these two antibiogram methods are quite similar and the accuracy of the data in disk diffusion method is still reliable [18].

Although, in this study, we encountered a very high resistance of *A. baumannii* which is still identifiable by the disk diffusion method and the E-test seems to be a better method for assessment of the microbial resistance, our study showed that the disk diffusion method also yields similar results and can be still used for this purpose.

For treating *A. baumannii* and its associated diseases, it is suggested to use stronger and newer generations of other antibiotics in order for treatment of the patients.

Perhaps, the main finding of this study was the very high resistance of this bacterium observed in Kerman and feeling the necessity of thinking of some strategies and solutions for reducing that microbial resistance as well as paying more attention to the selective treatments, antibiotic treatment course duration, and other instances that should be taken into account in any antibiotic diet in order to prevent and avoid such high levels of microbial resistance in our country.

At the end, it is recommended to conduct similar studies on other dangerous hospital microbes in hospitals of Kerman Province, Iran to find better methods for identification of the microbial resistance and its level in order to achieve a more appropriate pattern for microbial resistance and find proper methods for dealing with them.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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