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# Assessment of disk diffusion and E-test methods to determine antimicrobial activity of cefalotin and vancomycin on clinical isolates of *Staphylococcus aureus*

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**ABSTRACT**

*The present investigation aims to find a specific method to test the clinical isolates of Staphylococcus aureus in terms of antimicrobial susceptibility and resistance to two drugs of cefalotin and vancomycin using E-test and Disk Diffusion methods. Totally 80 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 vancomycin antibiotic, the S. aureus specimens were reported to be 15% resistant, 27% intermediate, and only 38% susceptible. Also, for cefalotin antibiotic, the specimens were reported as 48% resistant, 19% intermediate, and only 13% susceptible. In the E-test method for vancomycin antibiotic, the S. aureus specimens were reported to be 29% resistant, 18% intermediate, and 33% susceptible. Also, for cefalotin, the specimens were reported to be 59% resistant, 11% intermediate, and 10% susceptible. By statistical comparison using SPSS software and Chi Square test, the P-value of 0.001 was obtained; therefore, regarding the zero hypothesis, there is a significant relationship between these two methods of microbial susceptibility determination at ($p < 0.05$), and accept that the E-test method is still a suitable test for susceptibility determination for this bacterium. 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.*

**Keywords**: *Staphylococcus aureus*; antibiotic; resistance; disk diffusion; T-test

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## INTRODUCTION

*Staphylococcus aureus* as a gram-positive coccal bacterium is a prevalent human pathogen causing serious infections in hospitals all around the world. *S. aureus* is present in the nose of nearly 30% of healthy adults and on the skin of about 20%. *S. aureus* is also responsible for many forms of infections including superficial skin lesions (boils, styes), osteomyelitis, endocarditis, andurinary tract infections, especially in girls [1,2].Since two previous decades, both community-associated and hospital-acquired infections with *S. aureus* have increased. On the other hand, this increase has been accompanied by a rise in antibiotic-resistant strains especially, methicillin-resistant *S. aureus* (MRSA) and, more recently, vancomycin-resistant strains [3]. Nowadays, *S. aureus* can demonstrate better than other human bacteria the adaptive development of bacteria in the antibiotic response; so that it demonstrated a exceptional capability to rapid respond to each novel antibiotic with the expansion of a resistance mechanism, preliminary with penicillin and methicillin, to recently, linezolid and daptomycin [4]. According to the previous studies, the main resistance mechanisms of *S. aureus* are (i) enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), (ii) modification of the target with reduced affinity for the antibiotic

(notable examples being penicillin-binding protein 2a of methicillin-resistant S. aureus and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (for vancomycin and possibly daptomycin) and efflux pumps (fluoroquinolones and tetracycline) [5-7]. Due to *S. aureus* is one of the main agents of infection in the Infectious Diseases Ward, ICU, and other hospital wards, the injective cefalotin and vancomycin 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 [8].

To date, 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 [9]. 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 [10, 11].The present study aims to find a specific method to test the clinical isolates of *S. aureus*in terms of antimicrobial susceptibility and resistance to two drugs of cefalotin and vancomycin using E-test and Disk Diffusion methods.

## MATERIALS AND METHODS

### *Bacterial isolates*

Totally 80 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 a new and 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 $1.5\times10^8$ microorganisms/ml.

### *Microbial suspension transfer in disk diffusion method*

In sterile conditions, 50 $\mu l$ of the standardized microbial suspension ($7.5\times10^6$ microorganisms) was taken by a sample and poured into the test tubes containing 1ml of the sterile normal saline. The final microbial concentration ($7.5\times10^4$ 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 hours, 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.The0.5 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 [12].

**Table 1.Standarddiameter of inhibition halo for Cefalotine and Vancomycinein disk diffusion method**

| Antibiotic | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Vancomycine | ≥ 18 mm | 15-17 mm | ≤ 14 mm |
| Cefalotine | ≥ 48 mm | 14-19 mm | ≤ 13 mm |

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

The method of culture medium preparation is bacterial suspension and incubation. 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)[12].

**Table 2. Standard MICs for Cefalotine and Vancomycinein E-test method**

| Antibiotic | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Vancomycine | $\leq_{59\ \mu g/ml}$ | $\mu g/ml^{11}$ | $_{\mu g/ml}10\leq$ |
| Cefalotine | $_{\mu g/ml}\geq 39$ | $_{\mu g/ml}33$ | $_{\mu g/ml}18\leq$ |

***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 considered as the significant statistical difference.

## RESULTS

***Collected samples***

In this study, 80 *S. aureus* isolates were collected from 80 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 80 isolated specimens identified as *S. aureus* should be the same (Table 3). The collected specimens comprised of52 (65%) males and 28 (35%) females with average age of 41±13.1 years old.

**Table 3. Frequency of collected specimens**

| Specimen type | Number (%) |
|---|---|
| Trachea | 8 (10) |
| Urine | 3 (3.75) |
| Blood | 7 (8.75) |
| Wound culture | 40 (50) |
| Ascites fluid | 6 (7.5) |
| Cerebrospinal fluid | 16 (20) |
| Total | 80 (100) |

***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).The obtained results showed for cefalotin antibiotic, the *S. aureus* isolates were reported to be 48% resistant, 19% intermediate, and only13% susceptible. Also, for vancomycin antibiotic, the specimens were reported as38% resistant, 27% intermediate, and only 15% susceptible.

**Table 4. Results of disk diffusion method**

| Antibiotic | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Cefalotine | 13% | 19% | 48% |
| Vancomycine | 38% | 27% | 15% |

***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 cefalotin antibiotic, the *S. aureus* isolates were reported to be 59% resistant, 11% intermediate, and 10% susceptible. Also, for vancomycin, the isolates were reported to be 29% resistant, 18% intermediate, and 33% susceptible.

**Table 5. Results of E-test method**

| Antibiotic | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Cefalotine | 10% | 11% | 59% |
| Vancomycine | 33% | 18% | 29% |

***Comparison of results obtained for Ciprofloxacin in two methods***

The obtained results for cefalotine and vancomycine in two methods of disk diffusion and T-test showed that after performing the statistical Chi square test at confidence level of 95%, the P value for these two antibiotics was obtained ($p<0.001$) 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 (8, 13); 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 (14). 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 (15).The study aims to explore 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 *S. aureus* which causes some prevalent hospital infections and is often treated by cefalotin and vancomycin and other antibiotics of the same family. Eighty samples, with proved *S. aureus* were isolated from the ICU of General Hospitals of Kerman Province, Iran between November 2013 to April 2014.

The collected specimens included tracheal secretion (10%), urine (3.75%), blood and catheter (8.75%), wound culture (50%), ascites fluid (7.5%), and spinal fluid (20%).

Regarding the sites of specimen collection, it can be said that 50% of the cases were related to the wound infections caused by *S. aureus*, 20% to the CSF infections such as meningitis, 10% to the urinary tract infections, 8.5% to the blood stream infections, and 7.5% to the abdominal infections 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 vancomycin in disk diffusion and E-test methods was reported to be 15% and 29% resistant, respectively; while, previous study conducted in Northwest of Iran using the E-test method had reported the microbial resistance to be 20%, which indicates the higher prevalence of the microbial resistance of *S. aureus* against vancomycine antibiotic in Iran.

Results obtained for cefalotin demonstrate that the microbial resistance is very high in Iran so that, for this antibiotic, the microbial resistance was reported to be 48% and 59% in the disk diffusion method and in E- test, respectively.

By statistical comparison using SPSS software and Chi Square test, the P-value of 0.001 was obtained; therefore, regarding the zero hypothesis, there is a significant relationship between these two methods of microbial susceptibility determination at ($p<0.05$),and accept that the E-test 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 [16].

Although, in this study, we encountered a very high resistance of *S. aureus* 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 *S. aureus* 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 Interests**

The authors declare that there is no conflict of interests in this study.

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