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Susceptibility of Hospital Staphylococcus aureus Isolates Against Cephalosporins Using Manual E-test

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ABSTRACT

Thirty clinical isolates of *Staphylococcus aureus* were collected from different hospitals in Khartoum State. In vitro studies were carried out by susceptibility disk diffusion method against cephalosporins, (cefazolin example of the 1st -generation, cefuroxime example for the 2nd, and cefotaxime example of 3rd –generation). The E-test was then used to perform the susceptibility tests on the isolated strains for the same antibiotics, which was performed manually and evaluated with standard organisms. Minimum inhibitory concentration (MIC) was determined for each isolated strain. The E-test results were compared with the susceptibility disk method. Comparison of the results was made after converting the data to qualitative categories (susceptible, intermediate, and resistant). Disk diffusion method results, showed that 17% of isolated strains were resistant, and 83% were sensitive to each cefazolin, cefuroxime and cefotaxime. E-test showed the same results with cefazolin, but it differs with cefuroxime since 73%,7% and 20% were susceptible, intermediate, and resistant respectively, and 76%,7%and 17%, were susceptible, intermediate, and resistant to cefotaxime respectively. The analysis of the results proved that there was a significant difference between the three generations in zone inhibition and MIC values (P value <0.05). The average of MIC values for each antibiotics cefazolin, cefuroxime, and cefotaxime were (0.9-3.5-4.7) µg/ml, respectively. Cefazolin was the most potent one showing significant antimicrobial activity against isolated *S. aureus* strains. The average of zone inhibition values for each antibiotics cefazolin, cefuroxime, and cefotaxime were (35.1- 32.1 -30) mm, respectively. Also the significant inversely weak relationship was demonstrated between zone diameter and MIC for each antibiotics (r= -0.54). The technique of applying several disks with different antibiotic concentrations at the same time demonstrated clearly the minimum inhibitory concentration of the antibiotic. E. test also proved its significance over the single disk method.

المستخلص

ثلاثين حالة عزل سريري لبكتيريا المكورات العنقودية الذهبية تم جمعها من مختلف المستشفيات في ولاية الخرطوم حيث أجريت لها اختبارات الحساسية في المختبر ضد أجيال مضادات السيفالوسبورين الحيوية ، و استخدم السيفازولين كمثال للجيل الأول ، سيفوروكسيم كمثال للجيل الثاني، والسيفوتاكسيم كمثال للجيل الثالث . ثم تم استخدام (E-test)لأداء اختبار الحساسية على السلالات المعزولة للمضادات الحيوية أعلاه ، حيث تم اجراءه يدويا في المختبر ومعايرته بالبكتيريا العنقودية الذهبية القياسية .حيث تم تحديد الجرعة الأقل لتثبيط نمو البكتيريا لكل السلالات المعزولة ، وأيضا تم اجراء اختبار الحساسية لنفس السلالات المعزولة عن طريق اختبار نشر القرص.و مقارنة النتائج بين الاختبارين بعد تحويل البيانات إلى فئات نوعية(حساسة ، وسيطة ، ومقاومة).

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وأظهرت النتائج ، في طريقة نشر القرص ، أن 17٪ من السلالات المعزولة كانت مقاومة ، و 83٪ كانت حساسة لكل أجيال السيفالوسبورين على السواء .وأظهر اختبار (E-test)، نتائج مماثلة مع سيفازولين ، ومختلفة مع سيفوروكسيم 73٪ كانوا عرضة ،7٪ وسيطة ,و 20٪ ومقاومة ، وأيضا مختلفة مع السيفوتاكسيم، 77٪ كانت عرضة ،7٪ وسيطة ، و 17٪مقاومة. كما تم تحليل النتائج احصائيا والتي أثبتت أن هناك فرق احصائي للسلالات المعزولة بين الأجيال الثلاثة فى قياس قيم طول القطر والجرعة الاقل لتثبيط النمو MIC،بدلالة احصائية قيمة ($P < 0.05$). وكان متوسط قيم (MIC) لكل المضادات الحيوية سيفازولين ، سيفوروكسيم، والسيفوتاكسيم (0.9-3.5-4.7) ميكروغرام /مل ، على التوالي .سيفازولين كان أقوى نشاط ضد سلالات بكتريا المكورة العنقودية الذهبية المعزولة ، وكان متوسط قيم طول القطر لكل المضادات الحيوية سيفازولين 35.1 ملم ، سيفوروكسيم32.1 ملم ، والسيفوتاكسيم 30 ملم. أيضا اثبتت التحاليل ان هناك علاقة عكسية ضعيفة بين طول القطر والجرعة الاقل لتثبيط النمو لكل المضادات الحيوية ($r = -0.54$). أثبتت تقنية وضع المضاد الحيوي في أقراص بتركيزات مختلفة في الوقت نفسه في تحديد تركيز الحد الأدنى لتثبيط النمو بشكل واضح .و أيضا أكدت فعاليتة أكثر من طريقة القرص الواحد.

***KEYWORDS*:** E-test: Epsilometer test, (MIC) Minimum inhibitory concentration

INTRODUCTION

Over thirty species of the genus *Staphylococcus* can infect humans, but most important is *Staphylococcus aureus*. Microbial drug resistance is an inescapable consequence of the use of antimicrobial agents. As such their use is to subject the problems associated with microbial resistance. The study was continuous carried out to show the resistance of hospital *Staphylococcus aureus* strains to cephalosporin generations. *Staphylococcus aureus* causes serious disease and nosocomial infections[1]. E-test is a relatively new approach developed to test antimicrobial susceptibility and it is considered a simple and accurate susceptibility method for the emerging need to test most pathogenic organisms. And it is one possible solution to the problem of inappropriate antibiograms. E-test is a predefined, stable gradient of 15 antibiotic concentrations on a plastic strip. Using innovative dry chemistry technology and it is used to determine the on-scale (MIC) of antibiotics. E-test method gave an MIC result and was affected by test conditions in a similar way to other MIC and diffusion methods: Previous work has shown that cefazolin is a first generation cephalosporin was determined against *Staphylococcus aureus* isolates and showed that 17% of isolated strains were resistant, and 83% were sensitive to cefazolin[2]**.** The activities of cefuroxime was highly active against oxacillin-susceptible *Staphylococcus aureus*; however, activity against oxacillin-resistant isolates was more variable, the MICs 90% of the cefuroxime tested against oxacillin-resistant *S. aureus* were greater than 16, ug/ml[3]. Third generation cephalosporin, cefotaxime was highly effective against Gram positive and Gram negative bacteria. cefotaxime was active against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*[4]. The objective of the present study was to obtain (MIC) of cephalosporin antibiotics against *Staphylococcus aureus* by using in-vitro manual (E-test) and to compare and evaluate the performance with disk diffusion agar method.

MATERIALS AND METHODS

The study wsa conducted on *Staphylococcus aureus* strains which were isolated from sudanese hospitalized patient in Khartoum state. Identification, morphological and biochemical characterization of bacterial isolated colonies, after purification were initially Gram-stained by using Bergey's manual of determinative bacteriology[5]. The isolates were biochemically characterized and identified up to species level by performing various biochemical tests; β-Hemolysis, Coagulase test, Catalase test, and DNase test[5]. Disc diffusion (Bauer kirby) susceptibility test each *S. aureus*

isolates were performed on Mueller Hinton agar as growth medium. Homogenize well isolated *S. aureus* strains colonies in 0.85% NaCl to achieve 0.5 McFarland´s turbidity. Dip a sterile, non-toxic swab into the inoculum suspension and press the swab against the inside of the tube. The entire agar surface was evenly streaked, rotating 90° in three directions. Excess moisture was allowed to absorb for about 10-15 minutes so that the agar surface is completely dry before applying the Commercial Cephalosporin antibiotic Discs. Plates were inverted and placed in an incubator set to 35°C within 15 minutes after the discs were applied. After (16-18) hour's zones of inhibition of bacterial growth was measured across discs, and compared with those in the National Committee for Clinical Laboratory Standards[6].Results obtained were reported as resistant, intermediate or susceptible. The Paper was Art Paper type, normally, china clay (kaolin) coated on both sides of the paper was used. The finish of both sides was same, be it matt, were selected for preparing the disks. The selections were based on its ability to uniformly absorb sufficient volumes of antibiotic solution. Disks prepared by using an ordinary office two-hole puncher, paper disks with approximate diameter of 6.3mm. were punched out one by one from a sheet of paper, Precautions were taken to avoid overlapping of holes, Since the paper disks had a tendency to curl after punching, these were flattened by spreading them in a single layer on a clean smooth surface then pressed by rolling a bottle repeatedly. The disks were placed in a Petri dish then autoclaved for 15 minutes at 15 lbs pressure and allowed to cool[7]. Preparation of standard antibiotic powders: Cefazolin,Cefuroxime and Cefotaxime which provided from Federal Pharmacy and Board General Secretariat (National Drug Quality Control Laboratory), standard antibiotic powders were accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile volumetric flask.. Stock solution can be prepared using the formula according to National Committee for Clinical Laboratory Standards. (Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically).

Potency of antibiotic given by the manufacturer /1000 x Volume in ml required x Final concentration of solution = Weight of the antimicrobial

The concentrations of the antibiotic solution expressed in mcg/ml were based on the potency per disk. Different 10 concentrations were obtained for the three antibiotic solutions (two- fold) serial dilutions, 64 µg/ml were prepared from the stock of each antibiotic solutions, then serial double dilution was prepared for the following nines tubes. The continuous gradient covered a concentration range corresponding to 10 two-fold dilutions in a conventional dilution procedure[7]. Using the immersion method blank sterile prepared disks were soaked in the following concentration of each antibiotics; (64,32,16,8,4,2,1,0.5,0.25,0.13) µg/ml, then the impregnated disks were transferred into sterile Petri dishes and labeled with their defined concentration[8]. Without covering the Petri dishes, the disks were allowed to dry in a clean incubator at35°C for 2-3 hours[7]. After drying 50 to 100 disks were placed in small dark sterile air-

tight labeled containers with a desiccant at the bottom. A layer of sterile cotton or foam was placed over the desiccant to avoid contact with the disks. The disks were stored in a freezer at -14°C. Unopened containers were removed from the freezer 1 or 2 hours before use to equilibrate to room temperature before they were opened to minimize the amount of condensation that may occur when warm room air reaches the cold containers. The E-test utilizes circular disks with gradient concentration that has been impregnated with the antibiotics to be studied. A lawn of bacteria was inoculated on the surface of agar plate and by using forceps, beginning from the minimum concentration and upwards, and the gradient concentration disks were applied to the inoculated agar surface. Disks were in complete contact with the agar surface, which was located in one line beginning with the low concentration to the high one of the same antibiotics that the maximum concentration was nearest the rim of the plate. The antibiotics diffused out into the agar, producing an exponential gradient of the antibiotics to be tested. There was an exponential scale printed on the disks strip. After 16 hours of incubation, an elliptical zone of inhibition was produced and the point at which the ellipse met the defined disk concentration gave a reading for the minimum inhibitory concentration (MIC) of the antibiotics. The Performance of the Test first batch of locally produced disks were tested after preparation to determine if the inhibition produced fell within the limits set by WHO or NCCLS for the control organisms *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923. Initial testing was done three times, then two times every batch. A selected batch of commercially prepared disks and blank disks were used simultaneously blank disks were tested for presence of inhibitory activity[8].

Quality Control in Antibiotic Susceptibility Testing:

QC is performed to check the quality of medium, the potency of the antibiotic, to check manual errors. Quality control strains should be included daily with the test. Not more than 1 in 20 results should be outside accuracy limits. No zone should be more than 4standard deviations away from midpoint between the stated limits.

Control strains:

In all methods the use of control strains is required to ensure that the method is performing correctly, and both BSAC and NCCLS standardized methods include recommendations for susceptible and resistant control strains. In addition, participation in an external quality assessment scheme will provide an independent assessment of performance. The MICs of cefazolin, Cefuroxime, and Cefotaxime for the reference strains were within acceptable ranges listed by the National Committee for Clinical Laboratory Standards (NCCLS) (Table 1& 2).

Table 1: Reference strains result in Disk diffusion technique:

Organism	Zone inhibition diameter/mm		
	Cefazolin $(CZ)_{30\mu g}$	Cefuroxime (CU) 30µg	Cefotaxime (CE) 30µg
Staphylococccus aureus ATCC 25923	32	32	30
	Rang (29-35)	Rang (27-35)	Rang (25-31)

Table 2: Reference strains result in E-test technique:

Organism	MIC mcg/ml		
	Cefazolin	Cefuroxime	Cefotaxime
Staphylococccus aureus ATCC 29213	0.25	1	2
	Rang (0.25-1)	Rang (0.5-2)	Rang (1-4)

RESULTS

The present work consisted of 30 clinical isolates of *Staphylococcus aureus* which were tested against cephalosporin generations using disk diffusion agar method and E-test method, did not show any differences between the three generations in the susceptibility test with disk diffusion method. All of which showed 83% sensitivity and 17% resistance (Table 3), Cefazolin was the most active against isolated *Staphylococcus aureus,* with MIC ranging from 0.25-8µg/ml, MIC mean =0.93 µg/ml (Table 6). Cefuroxime was active against isolated *Staphylococcus aureus,* but less than cefazolin, the observed MIC of cefuroxime was ranging from 0.5-4 µg/ml, MIC mean =3.5 µg/ml (Table 6), which 20% strains were resistant, 7% intermediate, and 73% were sensitive to cefuroxime (Table 4).

Table 3: Susceptibility disk interpretation frequencies of S.aureus strains among the three generations of Cephalosporin

interpretation*	Cefazolin(CZ)		Cefuroxime(CU)		Cefotaxime(CE)		Total
Sensitive(S)	25	83 %	25	83 %	25	83 %	75
Resistant(R)	5	17 %	5	17 %	5	17 %	15
Total	30	100 %	30	100 %	30	100 %	90

Table 4: E-test values interpretation frequencies between the three generations of Cephalosporin

Interpretation	Cefazolin		Cefuroxime		Cefotaxime		Total
Sensitive	25	83 %	22	73 %	23	76 %	70
Intermediate	0	0 %	2	7 %	2	7 %	4
Resistant	5	17 %	6	20 %	5	17 %	16
Total	30	100 %	30	100 %	30	100 %	90

Table 5: Summarized zone diameter values of sensitive S.aureus strains against the three antibiotics

Antibiotics	No of *S.aureus*	Mean	Std. Deviation	Minimum	Maximum
Cefazolin	25	35.080	3.2650	27.0	41.0
Cefuroxime	25	32.120	3.1268	25.0	37.0
Cefotaxime	25	30.040	3.5879	23.0	38.0

Table 6: Summarized MIC values of sensitive S.aureus strains against the three antibiotics:

Antibiotics	No of *S.aureus*	Mean	Std. Deviation	Minimum	Maximum
Cefazolin	25	.9300	1.51815	.25	8.00
Cefuroxime	22	1.7500	.89642	.50	4.00
Cefotaxime	23	3.0000	1.83402	1.00	8.00

Cefotaxime, was active against isolated *Staphylococcus aureus* but less than cefazolin. The MIC showed a wide range from 1-32 µg/ml between the isolated strains with MIC mean = 4.7 µg/ml (Table 6), which 17% strains were resistant, 7% intermediate, and 76% were sensitive to cefotaxime (Table 4). Analysis showed significant differences in zone diameter between the three generations in disk diffusion agar method and also it showed significant differences in MIC values between the three Cephalosporin generations (P value≤0.05).The relationship between zone diameter and MIC values of the sensitive isolates was analyzed and showed inversely poor relationship between them, and the correlation equation of the independent variables MIC and dependant variables zone diameter; Y=34.7-0.76X (r= -0.54).

Figure 1: MIC of the three Cephalosporin generations againstS.aureus ATCC 29213

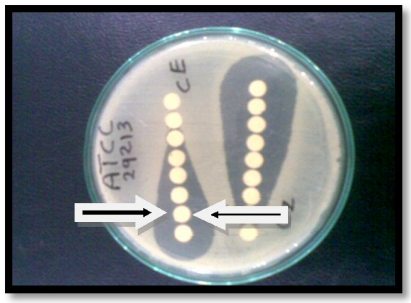

Figure 2: MIC of Cefazolin and Cefotaxime against S.aureus ATCC 29213 showing the ellipse zone, arrows show the MIC prickpoint

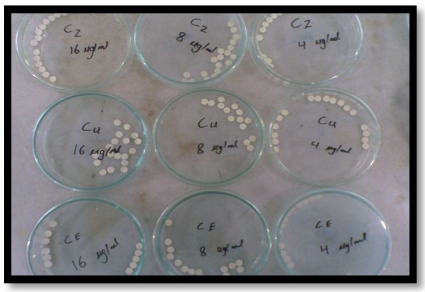

Figure 3: Show the disks impregnated with different concentrations of Cephalosporin's which performed in the lab

DISCUSSION

The 30 clinical isolates of *Staphylococcus aureus* were tested by *E-Test and disk diffusion agar methods.* Cefazolin showed high activity against *S. aureus,* more than 83% strains of *S.aureus* were sensitive to cefazolin with MIC mean 0,9 µg/ml ranging from 0.25-8 µg/ml. This result is similar to previous work showed cefazolin to be very active against *Staphylococcus aureus* isolates that 17% of isolated strains were resistant, and 83% were sensitive to cefazolin with MIC mean 1.1 µg/ml ranging from 0.25-8 µg/ml[2]. Cefuroxime showed less activity than cefazolin against *S. aureus,* the MIC values at 50% of *Staphylococcus aureus* isolates MIC_{50}=2 µg/ml, and at 90% of the isolated strains MIC_{90}=3.4 µg/ml, with MIC mean 3.5 µg/ml ranging from 0.5-4 µg/ml, this result disagree to previous studies that reported The in vitro activity of Cefuroxime was highly active against oxacillin-susceptible *Staphylococcus aureus*, the MIC values at 50% of *Staphylococcus aureus* isolates MIC_{50}=1 µg/ml, and at 90% of the isolated strains MIC_{90}= 1 µg/ml, with MIC mean 1 µg/ml ranging from 0.5-8 µg/ml, however, activity against oxacillin-resistant isolates was more variable, with MIC ranges extending from the lowest to above the highest concentrations tested(0.5-16)µg/ml. The MICs 90% of all of the cephalosporins tested against oxacillin-resistant S. aureus were greater than 16 ug/ml[3]. Cefotaxime showed less activity than both cefazolin and cefuroxime, During the present study, the MIC of cefotaxime was observed at 50% of *S.aureus*; MIC_{50}=2 µg/ml and 90% MIC_{90}=6.4 µg/ml, and MICs mean 4.7 µg/ml ranging from 1-8µg/ml, this result disagree to previous studies that showed cefotaxime was highly effective against Gram positive and Gram negative bacteria. cefotaxime was active against Staphylococcus aureus, the mean values for the MICs 50% of cefotaxime were 1.1-1.9 microgram/ml[9].

CONCLUSIONS

We concluded that the use of the E-Test is easier and more practical than use of the disk diffusion agar method. However, the higher cost of the E-Test method would likely discourage most laboratories to selecting it over disk diffusion for routine antimicrobial susceptibility testing of pathogenic bacteria. E-Test results appeared to be more reproducible than those obtained by the disk diffusion agar method. E-Test results changed less frequently when 30 randomly selected isolates were retested by both methods. The E-Test allowed for the simultaneous testing of four antimicrobial agents per 150-mm agar plate, and the same MÜeller-Hinton agar plates used for disk diffusion testing can be used for the E-Test.

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