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EVALUATION OF SUSCEPTIBILITY TESTING BY COMPARISON OF BROTH MICRODILUTION AND DISK AGAR DIFFUSION TESTS IN STAPHYLOCOCCI

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Abstract

There are methods for susceptibility testing for microorganisms that are isolated from different clinical samples. In order to obtain reliable results, many researchers have been working on the sensitivity of susceptibility tests and comparing the different methods.

Staphylococci are important bacteria that commonly come out as causative agents in many infections with both hospital and community origin. Accurate breakpoint of Staphylococcus aureus is crucial in the management and treatment of both colonized and infected patients.

To determine the in-vitro activity of trimethoprim-sulphamethoxazole, penicillin, tetracycline, vancomycine and erythromycin agents against S.aureus, isolates were collected from Naşide Halil Gelendost Primary School students of ages 7-12 were studied with microdilution and disk diffusion method. After comparison of the two methods results according to NCCLS criteria, in the 65 strains 97.5% correlation was determined.

Key words: *Susceptibility tests, Disk diffusion method,Microdilution methods*

Stafilokoklarda Sıvı Mikrodilüsyon ve Disk Difüzyon Yöntemlerinin Kıyaslanmasıyla Duyarlılık Testlerinin Değerlendirilmesi

Klinik örneklerden izole edilen suşların duyarlılıklarının araştırılmasında kullanılan çeşitli metotlar vardır. Güvenilir sonuçların elde edilmesinde infeksiyon etkeni olan mikroorganizmalar için duyarlılık testlerinin hassasiyeti halen birçok araştırıcı tarafından araştırılmakta ve farklı yöntemlerin kıyaslamaları yapılmaktadır.

Stafilokoklar, hem hastane infeksiyonu hem de toplumsal kaynaklı birçok infeksiyona neden olan önemli bakterilerdir. Hem kolonize hem de infekte hastalarda Staphylococcus aureus' un duyarlılık aralığı tanıda ve tedavide oldukça önemlidir.

Naşide Halil Gelendost ilköğretim okulundaki 7-12 yaşlar arasındaki öğrencilerden izole edilen S.aureus izolatlarına karşı trimetoprim-sulfametoksazol, penisilin, tetrasiklin, vankomisin ve eritromisin ajanlarının in-vitro aktivitesi mikrodilüsyon ve disk difüzyon yöntemleriyle araştırılmıştır. Bu iki yöntem sonuçları, NCCLS kriterlerine göre kıyaslandığında 65 izolat arasında % 97.5 oranında uyum görülmüştür.

Anahtar Kelimeler: *Duyarlılık testleri, Disk difüzyon yöntemi, Mikrodilüsyon yöntemi*

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Introduction

Methods used in the routine laboratory to test activity of antimicrobials include agar dilution, disk agar diffusion and various dilution methods. Methods for susceptibility testing should follow internationally agreed standards, unless there are specific motives for using national standards. The standardized disk agar diffusion method is the most common laboratory procedure used to determine in-vitro susceptibility of clinical isolates to antimicrobial agents, although technically more complex, broth dilution method may be used for the same purpose. In order to obtain reliable results, many researchers have been working on the sensitivity of susceptibility tests and comparing different methods (1-6).

The aim of the study was to evaluate the two susceptibility methods, disk agar diffusion and microdilution, for accurate breakpoint of *Staphylococcus aureus*. Staphylococci are presently one of the important isolates that commonly come out as causative agents in many infections and it is reported by several authors that these isolates may give unreliable results with different methods of various agents (1-6).

This report presents some results of the antimicrobial susceptibility obtained when Staphylococci isolates were tested by comparing both the microdilution and disk diffusion methods using trimethoprim-sulphamethoxazole, penicillin, tetracycline, vancomycine and erythromycin agents.

Experimental

Microorganisms

Total 65 isolates of *Staphylococcus aureus* collected from the students of ages 7-12 of Naşide Halil Gelendost Primary School.

Identification

The microorganisms were isolated on Mannitol Salt Agar (MSA)(Merck) medium. The opaque, white or cream colonies were detected on the agar surface and investigated microscopically. Gram-positive cocci occurring singly in pairs, tetrads or irregular clusters were determined as they could be Staphylococci. For the further investigation these colonies were adapted to the catalase test and catalase positive organisms were defined as Staphylococci. Coagulase positive Staphylococci that were grown on MSA surface were defined as *S.aureus* (7).

Inoculum preparation

Each isolate grown overnight on MSA at 37C, was suspended in Mueller Hinton Broth (MHB)(Merck) medium and vortexed thoroughly to achieve a homogen suspension. Turbidity was adjusted to the density of 0.5 McFarland macroscopically. This suspension (10^8 cells/ml) was used for each method of susceptibility testing.

Agents

Standard antibacterial powders of trimethoprim-sulphamethoxazole (**TMP-SMX**; Roche), erythromycin (**E**; Koçak), penicillin (**P**; Faco), tetracycline (**TE**; Sigma), vancomycine (**VA**; Lilly) were obtained from their respective manufacturers. The stock solutions of Trimethoprim-sulphamethoxazole, penicillin, tetracycline, vancomycine and erythromycin used in this study were dissolved in dimethylsulphoxide (DMSO)(Merck)(tetracycline, vancomycine), 95% ethanol (erythromycin) and water (trimethoprim-sulphamethoxazole, penicillin). Disks of trimethoprim-sulphamethoxazole (23.75μg **SMX**, 1.25μg **TMP**, Oxoid), erythromycin (15μg, Oxoid), penicillin (10 IV/IE, Becton Dickinson), tetracycline (30μg, Oxoid), vancomycine (30μg, BBL), and oxacillin (1μg, Oxoid) were used in this study.

Microdilution method

Microdilution technique was employed for the determination of MIC values with microplates 96-well Falcon^R (USA) microplates. Brinkman transferpette^R (Germany) was used for the two-fold dilution of the compound in the wells. The solutions of compounds were prepared at 128,……,0.063 μg/ml concentrations in the wells of microplates by diluting with media. The microorganism suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at the density of McFarland 0.5 (10^8 cfu/ml). Suspension of the microorganisms at 10^5 cfu/ml concentration were inoculated to the two-fold diluted solution of the compound. There were $5x10^4$ cfu/ml microorganism suspension in each well after inoculations. Mueller-Hinton Broth(Oxoid) was used for diluting the microorganisms suspension and for two-fold dilution of the compounds. DMSO, microorganism mixtures, pure microorganisms and pure media were used as control wells. The lowest concentration of the compounds that completely inhibits macroscopic growth was determined as Minimum Inhibitory Concentrations (MICs) were reported (8).

Disk diffusion testing

Agar diffusion method has been used for direct inhibition tests and these studies were performed using standardized inoculums with selective media. Disks were directly applied on the cultured plates. After incubation at 24 hours, zones of bacterial inhibition were measured in millimeters for all tested materials. MSA (Oxoid), MHB (Difco), MHA (Difco) were used to culture for *S.aureus*. *S.aureus* was incubated aerobically in MHB. Then 10μl of each culture was inoculated onto the agar plates to form a single colony. After incubation at 37^{0}C for 20 hours the microorganism suspensions used for inoculation were prepared by diluting fresh cultures at the density of McFarland 0.5 (10^8 cfu/ml). For determining the inhibition halos of the standardized bacterial cultures were spread-plated on each agar plate. Trimethoprim-sulphamethoxazole, penicillin, tetracycline, vancomycine and erythromycin were also used in the test and disks applied symmetrically on the surface of each type of agar with sterile forceps. Plates were incubated at 37 ^{0}C for 24 h. After incubation period, the agar plates evaluated and the zones of microbial inhibition were measured in millimeters. Media and microorganisms were used as control petri dishes (9).

Results and Discussion

Procedure

The breakpoints recommended by National Committee for Clinical Laboratory Standards were accepted for all Antimicrobial agents for which recommendation were made. The distribution of differences in MICs determined by reference microdilution an disk diffusion methods are presented in Table I. Agreement within one twofold dilution between disk diffusion test were 90.7 % for trimethoprim-sulphamethoxazole(**TMP-SMX**), 98.5 % for erythromycin(**E**), and vancomycine (**VA**) 100 % for penicillin (**P**) and **TE** (tetracycline).

62 (95.2 %) and 59 (90.7 %) isolates were found susceptible to **TMP-SMX** by microdilution and disk diffusion method respectively. 65 (100 %) isolates were found susceptible to **E** and **VA** by microdilution method; 64 (98.5 %) isolates by disk diffusion method. 61 (93.8 %) isolates were found susceptible to **P** and 63 (96.9 %) isolates were found susceptible to **TE** with either method. Oxacillin resistant isolates were also found resistant to penicillin.

TABLE 1. Distribution of differences in susceptibility of antimicrobial agents against 65 Staphylococci by disk diffusion versus microdilution methods

		MICs (mg/ml)	Disk(mm)	*S
TMP-SMX	S	(≤2) n=62 (95.4 %)	(24-32) n=59 (90.8%)	90.8 %
	R	(>2) n=3	(< 24) n=6	
E	S	(≤ 0.5) n=65 (100 %)	(22-30) n= 64 (98.5%)	98.5 %
	R	(> 0.5) n=0	(< 22) n=1	
VA	S	(≤ 4) n=65 (100 %)	(17-21) n=64 (98.5%)	98.5 %
	R	(> 4) n=0	(< 17) n=1	
P	S	(≤ 0.12) n=61(93.8%)	(26-37) n=61 (93.8%)	100 %
	R	(> 0.12) n=4	(< 26) n=4	
TE	S	(≤ 4) n=63 (96.92%)	(24-30) n=63 (96.92%)	100 %
	R	(> 4) n=2	(< 24) n=2	

*S: Susceptibility rates of disk agar diffusion and microdilution methods, R: Resistant, S: Sensitive, **TMP-SMX**: Trimethoprim-sulphamethoxazole, **E**: Erythromycin, **VA**: Vancomycine, **P**: Penicillin, **TE**: Tetracycline

Six isolates were found resistant to **TMP-SMX**, 1 isolate to **E** and **VA**, 4 isolates to **P** and 2 isolates to **TE** were found resistant with disk diffusion method.

Due to the satisfactory results against methicillin susceptible (61) and resistant (4) *S.aureus* isolates in-vitro, **TMP-SMX**, **E** and **VA** has to be confirmed for their in vivo activity when considering in the treatment of staphylococcal infection.

After comparison of the two methods according to NCCLS criteria, in the 65 strains 97.5 % correlation was determined. No disagreement between **P** and **TE** were observed when values of any of the two methods evaluated were compared.

The accuracy of antimicrobial susceptibility tests is a crucial step for the clinical management of patients with serious infections. Mendes et al.[10] have reported that antimic-

robial resistance has emerged in bacterial pathogens which include Staphylococcus spp. throughout the world in the rising problem of resistance detected by both national and international surveillance programs. They also have reported that, to evaluate the susceptibility test systems, it was necessary to determine the power of discrimination of various antimicrobial susceptibility test methodologies used by the participating centers, followed by the respective susceptibility test results (10).

For this reason in order to obtain reliable results; our report presents some of the results of antimicrobial susceptibility obtained for Staphylococci isolates when tested by comparison with both microdilution and disk diffusion methods using trimethoprim-sulphamethoxazole, penicillin, tetracycline, vancomycine and erythromycin agents.

When interpretive breakpoints are considered, the data of the study show good agreement between the two methods. In our research, results of *S.aureus* isolates gave good correlation compared with two methods of agar diffusion and microdilution for penicillin and tetracycline. However, the discrepancies observed between the microdilution and disk diffusion methods for erythromycin, trimethoprim-sulphamethoxazole and also vancomycine could be depended on the molecular structure.

Since erythromycin has a large molecular size, disk diffusion test may show decreased susceptibility for this agent for the tested isolates. So our data agrees with the findings of earlier workers who have reported that glycopeptides non susceptibility in Staphylococci need to be confirmed by determination of MICs (11). Similar research was studied by Martineau et al.[12], who have tested Staphylococcus spp. and determined a correlation of 98.5% for erythromycin, between microdilution method and MicroScran. After comparison of the two methods according to NCCLS criteria, in the 65 strains 97.5% correlation was determined in our study.

Similar to the findings of Kelly et al.[13], microdilution MICs of some *S.aureus* isolates were lower than those obtained by disk diffusion method. Our data agrees with the findings of this report. When comparing disk diffusion with microdilution method, discrepancies between two methods have been reported as 6.3% by Martinez et al.[14] for Coryneform bacteria. In our study 2.5% discrepancies were determined between two tested methods for Staphylococci.

Alborzi et al.[15] have studied with the same antibiotics that were used in our study and reported that the changing pattern of resistance of *S.aureus* would be wise to have a periodic surveillance of these changes once every 3 to 4 years. They have reported sensitivity as 100 % for vancomycine, likely with our study results for *S.aureus*.

Agar disk diffusion is the most used antibiotic susceptibility assay in clinical microbiology laboratories, but it is not particularly accurate because it may demonstrate a lack of reproducibility and sensitivity than methods are generally accurate of broth dilution (12).

In summary, microdilution test can be recommended for routine penicillin and tetracycline susceptibility testing of *S.aureus*, whereas for these microorganisms erythromycin, trimethoprim-sulphamethoxazole and vancomycine disk diffusion test can not be recommended because of susceptibility breakpoints.

Conclusion

All antibiotics used in this study, were active against Staphylococci isolates, so they should be used when the results of antibiotic sensitivity tests confirmed by other methods. Disk diffusion method can be used because of the practical difficulty of broth microdilution but it should be confirmed with broth microdilution if isolates were found resistant with disk diffusion methods.

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