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SUSCEPTIBILITY TESTING OF CLINICAL *STAPHYLOCOCCUS AUREUS* ISOLATES BY DIFFERENT METHODS AND STUDY OF ANTIBIOTIC RESISTANCE

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ABSTRACT

A total of 70 isolates were collected from neonates unit, Zagazig university hospital and identified to be coagulase-positive *Staphylococcus aureus*. The study was planned to evaluate and compare the methods of determination of minimum inhibitory concentrations for some antibiotics against *S. aureus* isolates. In disk diffusion method, the most effective drug was ciprofloxacin (77%) and the least effective was chloramphenicol (50%). The MIC range, MIC_{50} and MIC_{90} values by broth microdilution and agar dilution methods were closely related. The most effective drug against *S. aureus* isolates was ciprofloxacin then cefotaxime while, ampicillin/ sulbactam showed lower activity with higher MIC range, MIC_{50} and MIC_{90} than expected. E-test results showed high percentage of agreement with most of broth microdilution results and the difference was detected to be within the limits (±1-2 dilution). By using broth microdilution method as reference method and analysis of results by scattergram, very major discrepancies were found in ciprofloxacin (3.5%) and cefotaxime (1.5%). Major discrepancy was noticed only with rifampicin (3%) and minor discrepancies were detected in all of antibiotics except ampicillin/ sulbactam which showed 100% agreement and there are no discrepancies. The multidrug resistant isolates showed a plasmid DNA of sizes ranged from 8-30 kb with the transfer of ampicillin, cefotaxime, chloramphenicol and tetracycline resistance to the competent *E. coli* cells. In conclusion, E-test is considered efficient and simple method for rapid determination of antibiotic MIC and activity.

INTRODUCTION

Staphylococcus aureus is one of the most common pathogens and causes of community-acquired diseases and have been become a common cause of nosocomial infections, particularly bloodstream infections and infections related to prostheses. They account for about 9-10% of hospital-acquired infections[1,2]. Individuals identified with staphylococal infections are most commonly found in hospital intensive care, burns, dermatology and surgical units, reflecting the increased susceptibility of these individuals to staphylococal infections due to compromised immune function[3]. *S. aureus* most commonly causes a localized skin infection, although it can also infect the eye, nose, throat, urethra, vagina, and gastrointestinal tract. In addition, *S. aureus* can cause more serious ailments when it enters the bloodstream, such as pneumonia, osteomyelitis, arthritis endocarditis, myocarditis, brain abscesses and meningitis[4].

The appropriate treatment for an infectious disease requires the isolation of infectious agent and detection of its susceptibility or resistance to antimicrobial agents used in therapy. Different microbial species and strains have different degrees of susceptibility to different chemotherapeutic agents. Moreover, the susceptibility of a microorganism can be changed with time during therapy with a specific drug[5]. The antimicrobial susceptibility may be reported qualitatively as sensitive, intermediate or resistant or quantitatively in terms of the lowest concentration of the agent inhibits the growth (MIC) or kills (MBC) microorganism[6].

The conventional test of MIC is the broth dilution method, however, the most widely used method for testing microbial susceptibility to chemotherapeutic agents is the disk diffusion method of NCCLS[7]. Disk diffusion technique is useful for routine drug susceptibility test for bacteria because of simplicity and low cost. Moreover, disk diffusion method can be adapted to provide qualitative categories as susceptible, intermediate and resistant to different antimicrobial agents[3]. Fosola et al.[9] used disk diffusion technique accurately to obtain categorical susceptibility of *Streptococcus pneumoniae* for many non-lactam antibiotics. Epsilometer test (E-test) is a recent technique for quantitative determination of MIC on agar[8].

The study was planned as an attempt to achieve the isolation and microbiologically and biochemically identification of coagualse-positive *Staphylococcus aureus* isolates from clinical samples. Then detect susceptibility of the isolates by disk diffusion technique. The MIC will be determined by broth microdilution and agar dilution techniques and E-test. Comparison between MICs results of the isolates by the used three methods.

MATERIALS AND METHODS

Bacterial Isolates

Five hundred and eighty swabs from the buccal cavity, skin and incubators of neonates were obtained from Neonates Care Unit (NCU), Zagazig University Hospital, Egypt. The swabs were cultivated onto mannitol salt agar and blood agar at 37 °C for up to 2 days. The isolates were identified by API-20 Staph system (bioMéreuex, Marcy L'Eloite, France) and used for growth on nutrient broth containing 5 % NaCl, catalase and oxidase tests and biochemical characters. The isolates were tested by slide and tube-coagulase, DNase, and phosphatase tests, then mannitol and glucose fermentation[10, 11].

Antimicrobial susceptibility testing

Susceptibility tests were carried out by disk diffusion method on Müller-Hinton agar according to

NCCLS[7]. The isolates were tested against ampicillin/sulbactam (SAM, 20/ 10 µg), cefoparzon (CFP, 75 µg), cefotaxime (CTX, 30 µg), ceftriaxon (CRD, 30 µg), erythromycin (E, 15 µg), chloramphenicol (C, 30 µg), tetracycline (T, 30 µg), gentamicin (GN, 10 µg), ciprofloxacin (CIP, 5 µg), ofloxacin (OFX, 5 µg), and rifampicin (RD, 5 µg). The antibiotics disks are the product of Oxoid, Hamsphire, England. The diameter of inhibition zones were interpreted according to Koneman et al.[10] and NCCLS[7].

MIC determination

Preparation of inoculum

Accurately 100 µl from overnight culture were transferred aseptically onto 3 ml saline to obtain turbidity visually comparable to 0.5 McFarland equal to about 10^6 CFU/ ml[6].

Broth microdilution technique

Müller-Hinton broth was used for determining the MICs of antibiotics by microdilution method according to NCCLS[12]. In sterilized microtiter plates, a two fold serial dilution of antibiotics was carried out. The antibiotics concentrations ranged from 0.06 to 512 µg/ ml and next 2 wells were served as positive and negative control. From prepared bacterial inoculum, 100 µl was inoculated to each well except negative control (free from antibiotic and inoculum). The MICs were determined for ampicilin/ sulbactam (Unasyn, Pfizer, Egypt), cefotaxime (Cefotax, EPICO, Egypt), erythromycin (Erythrocin, Abbot, Egypt), rifampicin (Rimactan, Biochemie, Egypt), gentamicin (Garamycin, Glaxo Wellcome, Egypt), chloramphenicol (Cidocetine, Cid, Egypt), tetracycline (Tetracycline, ADCO, Egypt) and ciprofloxacin (ADCO, Egypt). The stock antibiotics concentrations were prepared by dissolving 512 mg from antibiotic in 100 ml medium and then serially two-fold diluted in the wells. The plates were incubated at 37°C for 16-20 h. The MIC_{50} and MIC_{90} of tested antibiotics were determined and interpreted according to NCCLS guideline[12].

Agar dilution method

Müller-Hinton agar was used and the antibiotics concentrations ranged from 0.06 to 512 µg/ ml were prepared. Each antibiotic concentration was inoculated into a Petri-dish and 10 µl from bacterial inoculum was loaded over the surface of agar in a separate square. The plates were incubated at 37°C for 16-20 h in humidified incubator before recording the results. The antibiotics used in broth microdilution method were used in agar dilution method with the same concentrations. The concentration of antibiotic that inhibits visible growth was considered the MIC and each antibiotic MIC was estimated according to NCCLS guideline[12].

E-test

Epislometer test strips (E-test, AB Biodisk, Solna, Sweden) used are mentioned in table 1. The strips were allowed to warm up to room temperature for 30 min then transferred onto the surface of Müller-Hinton agar inoculated with 100 µl from prepared bacterial inoculum. The plates were incubated overnight at 37°C and the elliptical zone of inhibition is produced. The MIC is read directly at the point of intersection of the zone of inhibition with the strip.

Table 1: Epislometer test and antibiotic gradient concentration in the strips

Antibiotic	Symbol	Range (µg/ml)
Ampicillin/ sulbactam	AB	0.016-256
Cefotaxime	CT	0.016-256
Choramphenicol	CL	0.016-256
Ciprofloxacin	CI	0.002-32
Erythromycin	EM	0.016-256
Gentamicin	GN	0.064-1024
Rifampicin	RI	0.016-256
Tetracycline	TC	0.016-256

Interpretation of Results

The results were compared by scattergram and NCCLS recommendation criteria. The discrepancy rates for antibiotics were calculated according to minor, major and very major error or discrepancy. A minor error is a one category difference between methods; such as an intermediate result obtained with reference method and susceptible or resistance with others. Major discrepancy occurs when the reference method shows susceptibility and the comparative methods show resistance. In contrast, very major discrepancy occurs when the reference method shows resistance and comparative methods show susceptibility. The MICs of broth microdilution method was used as a reference method as reported by Fuchs et al.[13].

Plasmid extraction

The plasmids were extracted from multidrug-resistant *S. aureus* strains by enzyme lysis method[14]. The cells were grown for 16-18 h at 37°C in LB broth. The cells were pelleted by centrifugation at maximum speed (14000 rpm) at 4°C for 2 min. The cell pellet was washed once with 1 ml saline then repelleted and resuspended in 100 µl saline. The cells suspension was mixed thoroughly with 10 µl lysostaphin enzyme (10 mg/ml, Böhringer, Germany) and incubated at 37°C with shacking for 30 min. The cells were collected by centrifugation at 14000 rpm for 30 seconds and resuspended in 100 µl solution I (100 mM glucose and 10 mM Tris.Cl, pH 8), then mixed with 200 µl solution II (1% SDS and 0.2 N NaOH) to give clear lysate. About 150 µl solution III (K. acetate 5 M and glacial acetic acid) was added, mixed well and kept in crushed ice for 30 min. The tubes were centrifuged at 4°C for 10 min at 14000 rpm. The supernatant (≈ 0.5 ml) was extracted once with phenol-chloroform solution and the clear aqueous layer was mixed thoroughly with 1 ml −20°C absolute ethanol and kept in crushed ice for 1 h. The tubes were centrifuged at 4°C for 5 min at maximum speed and the residue was washed once with −20°C 70% ethanol, air dried and dissolved in 100 µl TE buffer. RNase (2 µl from 10 mg/ml, Böhringer, Germany) was added to get rid of RNA. The extracted plasmids were electrophoresed in 0.8% w/v agarose

gel, visualized by UV-transilluminator after staining with ethidium bromide and photographed with Polaroid Camera. Plasmid undigested marker were purchased from Promega, California, USA and λ DNA digested with *EcoR*I and *Hind*III marker from Böhringer Mannheim, Germany. The plasmid transformation was carried out by thermal shock method using *E. coli* DH5αF competent cells according to Maniatis et al.[14].

RESULTS

Bacteriological Identification

A total of 75 isolates of Gram-positive cocci, arranged in clusters with yellow colonies on mannitol salt agar, and showing positive-catalase, phosphatase and DNase but negative-oxidase and ferment glucose and mannitol. The isolates were coagulase positive *S. aureus* except 5 isolates were coagulase-negative. The isolates were coagulase-positive *S. aureus* (CPS) and 5 isolates were coagulase-negative *Staphylococcus spp.* (CNS). These results were confirmed by API-20 Staph system.

Antibiotic susceptibility by disk diffusion method

The results of disk diffusion were shown in table (2) according to NCCLS[7]. The most effective drugs used against *S. aureus* isolates were ciprofloxacin (CIP), rifampicin (RD) and ofloxacin (OFX) and showed activity of 77, 70 and 69%, respectively. However, cefoperazone (CFP), ampicillin/ sulbactam (SAM), cefotaxime (CTX) were less effective and showed activity of 57%, against the tested isolates. Irregularly, gentamicin (GN) exhibited activity lower than the expected; about 55% while tetracycline (TC), erythromycin (E) and chloramphenicol (C) showed activity of 63, 60 and 50%, respectively, against the tested isolates.

Table 2: Antimicrobial susceptibility of *S. aureus* isolates to 11 antibiotics by disk diffusion method

Antibiotic	Disk content (µg/ml)	NCCLS standard (mm)			Tested *S. aureus* Results			Activity %
		R ≤	I	≤ S	R	I	S	
CFP	30	12	13-17	18	20	10	40	57
CIP	5	15	16-20	21	8	8	54	77
CTX	30	14	15-22	23	19	11	40	57
C	30	12	13-15	16	22	13	35	50
E	15	13	14-17	18	15	13	42	60
GN	10	12	13-14	15	22	9	39	55
OFX	5	16	17-24	25	15	7	48	69
RD	5	16	17-19	20	19	2	49	70
SAM	20	28	–	29	30	–	40	57
TC	30	14	15-18	19	18	8	44	63

R: resistant, I: intermediate and S: susceptible

MIC by Broth Microdilution Technique

The distribution of MICs of 8 antimicrobial agents against 70 *S. aureus* isolates by broth microdilution method is shown in tables (3 and 5). MICs of ciprofloxacin (CIP) and cefotaxime (CTX) are in the range from 0.125-16 and 0.125-64 µg/ml with 0.5 and 1 µg/ml are the most active concentrations, respectively. MICs of chloramphenicol (C) and tetracycline (TC) have a range from 2-128 and 0.5-128 µg/ml and the most effective concentrations are 4 and 2 µg/ml, respectively. A wide range of MIC was obtained with Erythromycin (E, 0.06-128 µg/ml), gentamicin (GN, 0.06-256 µg/ ml) and rifampicin (RD, 0.06-32 µg/ ml). The most active concentration is 0.25 µg/ml for E, GN and RD. Ampicillin/ sulbactam showed both MIC range of 0.5-64 µg/ ml and the most active concentration is 4 µg/ml.

MIC by agar dilution technique

There is no wide difference between MIC of the tested antibiotics against 70 *S. aureus* isolates by agar dilution method and broth microdilution method. Hence, broth microdilution MICs results were used for comparison and analysis. Tables (4 and 5) show the distribution for MICs of 8 antibiotics against the tested *S. aureus* isolates. CIP has MIC of about 0.125-32 µg/ml and 8 µg/ml is MIC_{90} in broth microdilution and agar dilution methods. But MIC_{50} is lower by one dilution in broth microdilution (0.5 µg/ml) than agar dilution (1 µg/ml). CTX has a wide range of activity (0.06-64 µg/ ml) and its MIC_{90} in both used methods is 16 µg/ ml but its MIC_{50} with the susceptible isolates is 0.5 and 2 µg/ml in broth and agar method, respectively. C and TC have MICs range from 1-256 and 0.5-128 µg/ml, in respective manner. The same values of MIC_{50} (2 µg/ml) and MIC_{90} (32 µg/ml) of TC in agar and broth methods were obtained and C showed also MIC_{50} of about 8 µg/ml in both methods but MIC_{90} by broth method is higher (64 µg/ml) by one dilution than agar dilution method (32 µg/ml). Like broth microdilution method, erythromycin, gentamicin and rifampicin have MIC_{50} of about 0.25 and 4, 2 and 1, and 0.5 µg/ml in both methods, respectively. But MIC_{90} of E, GN and RD, in respective manner were 16, 32 and 8, 16 and 8 µg/ml. Ampicillin/ sulbactam has MIC_{50} and MIC90 by agar dilution (2 and 8 µg/ml) lower than broth microdilution method (4 and 16 µg/ml) against the susceptible *S. aureus* isolates.

Scattergram analysis

It was done by plotting of zone diameter around the antibiotic disk against MICs by broth microdilution for individual isolates are shown in figure 1 (a-h). For ciprofloaxcin, the vertical and horizontal lines demonstrate the susceptibility and resistance breakpoints (Figure 1a). Except for 4 isolates, the results are in agreement between the two methods. Also, except for 4 isolates, cefotaxime showed agreement between the two methods (Figure 1b). In case of gentamicin and tetracycline (Figures 1c and 1d), showed agreement with 2 methods except for 2 isolates. For rifampicin (Figure 1e), the results are in agreement except for 3 isolates. For ampicillin/ sulbactam (Figure 1f), the results are in agreement and there was no discrepancy.

In case of chloramphenicol (Figure 1g), the results are in agreement for the two methods except for 2 isolates. For erythromycin (Figure 1h), the results are in agreement except for 4 isolates.

Table 3: Distribution of MIC s by broth microdilution technique of antibiotics against *S. aureus* isolates

Antibiotic	NCCLS			Number of isolates with MIC (µg/ml)												
	R	I	S	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06
CIP	4	2	1	–	–	–	–	5	4	–	8	13	23	15	2	–
CTX	2	1	0.5	–	–	1	5	2	1	3	–	25	12	11	7	3
C	32	16	8	–	5	12	5	6	15	17	9	–	–	–	–	–
E	8	2	0.5	–	2	2	–	5	8	7	4	–	2	20	8	12
GN	16	8	4	1	–	7	3	9	5	7	2	5	4	12	7	6
RD	4	2	1	2	–	–	3	4	4	5	1	13	12	12	8	6
SAM	8	–	4	–	3	10	8	9	–	10	15	4	2	–	1	–
TC	16	8	4	–	1	8	5	4	6	12	21	9	4	–	–	–

Table 4: Distribution of MIC s determined by agar dilution technique for antibiotics against *S. aureus* isolates

Antibiotic	NCCLS			Number of isolates with MIC (µg/ml)												
	R	I	S	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06
CIP	4	2	1	–	–	–	2	3	4	4	5	22	12	8	10	–
CTX	2	–	1	–	–	7	4	1	2	3	20	10	12	6	3	2
C	16	–	8	2	4	5	5	5	10	13	13	11	–	–	–	–
E	8	2	1	–	2	2	2	11	13	13	8	2	4	4	4	3
GN	16	8	4	–	1	3	2	4	2	12	9	15	14	7	4	2
RD	4	2	1	2	–	–	–	2	2	5	1	20	24	4	4	4
SAM	8	–	4	1	1	8	4	4	2	3	22	14	7	1	1	–
TC	16	8	4	–	3	7	5	24	15	7	1	2	4	–	–	–

Table 5: MIC range, MIC_{50} and MIC_{90} for 8 antibiotics by broth microdilution and agar dilution methods against *S. aureus* isolates

Antibiotic	MIC (µg/ml)					
	Broth microdilution			Agar dilution		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
CIP	0.125-16	0.5	8	0.125-4	1	8
CTX	0.06-64	0.5	16	0.06-2	2	16
C	2-8	8	64	2-8	8	32
E	0.06-8	0.25	16	0.06-8	4	16
GN	0.06-2	2	32	0.06-4	1	8
RD	0.06-1	0.5	16	0.125-256	0.5	8
SAM	0.125-2	4	16	0.125-4	2	8
TC	0.125-8	2	[illegible]	0.5-16	2	32

Analysis of the degree of discrepancies

The degree of discrepancies between disk diffusion and broth microdilution method of 70 *S. aureus* to 8 antibiotics including CIP, CTX, C, E, GN, RD, SAM and TC are shown in table (6). The results show complete agreement with 100% for SAM. The number of strains showing agreement for CIP, C and GN were 66 (94%), 68 (97%) and 67 (95.7%), respectively. However, CIP strains show with major and minor discrepancies in 4 strains (6%) where for C and GN, only minor discrepancies were obtained with 2 (3%) and 3 (4.3%) strains. For RD the number of strains that had agreement was 67 (95.7%) and major discrepancy in 2 strains (3%) and one strain with minor discrepancy (1.5%). In TC, two strains showed minor discrepancy (3%) with 98% agreement. In case of E and CTX, 94% from the tested strains was in agreement except 4 strains (6%) showed minor discrepancy in case of E. For CTX, very major discrepancy was shown in one strain (1.5%) and minor discrepancy was found in 3 isolates (4.5%).

Table 6: The degree of discrepancies in antibiotic susceptibility results between disk diffusion and microdilution method for *S. aureus* isolates

Antibiotic	Agreement		Very major discrepancies		Major discrepancies		Minor discrepancies	
	No.	%	No.	%	No.	%	No.	%
CIP	66	94	1	1.5	–	–	3	4.5
CTX	66	94	1	1.5	–	–	3	4.5
C	68	97	–	–	–	–	2	3
E	66	94	–	–	–	–	4	6
GN	67	95.7	–	–	–	–	3	[illegible]
RD	67	95.7	–	–	2	3	1	1.5
SAM	70	100	–	–	–	–	–	–
TC	68	97	–	–	–	–	2	3

E-test

A different degree of discrepancies result between disk zone diameter and MIC obtained by broth microdilution and agar dilution techniques was noted with strains for some antibiotics (Figure 2). These discrepant isolates were used to determine MIC by E-test to know the degree of agreement between the 3 methods (Table 7). From result it is noted that a high level of correlation was obtained by MIC from broth microdilution and E-test with essential agreement rates (± doubling dilution). Minor discrepancies obtained by one strain representing of 1.5% of isolates. MIC by E-test was one dilution less than that by broth microdilution for 5 isolates and was more than one dilution for one isolate and by more than 2 dilutions for one isolate.

Plasmids Characterization and Transformation

The plasmids of the multidrug resistant 13 *S. aureus* isolates were characterized and transformed to the competent cells of *E. coli*. The plasmids DNA showed sizes ranged from 8-30 kb (Figure 3). The isolate no. 2, 17 and 19 (lanes 1, 8 and 9) showed the largest plasmids of sizes about 30, 28 kb, respectively. However, isolates no. 9, 14 and 15 (lanes 5-7) revealed the smallest plasmids of sizes 9 and 8 kb, in respective manner. But the isolates no. 3, 4, and 7 (lanes 2-4) have closely related plasmids of sizes 14-15 kb, respectively. Lanes 10-13 of isolates no. 23, 24, 25 and 32 have plasmids of size about 12 kb. After plasmid DNA transformation, the competent cells of *E. coli* showed resistance to ampicillin, cefotaxime, chloramphenicol and tetracycline. This resistance was considered plasmid DNA-encoded but the others were chromosomal-encoded.

DISCUSSION

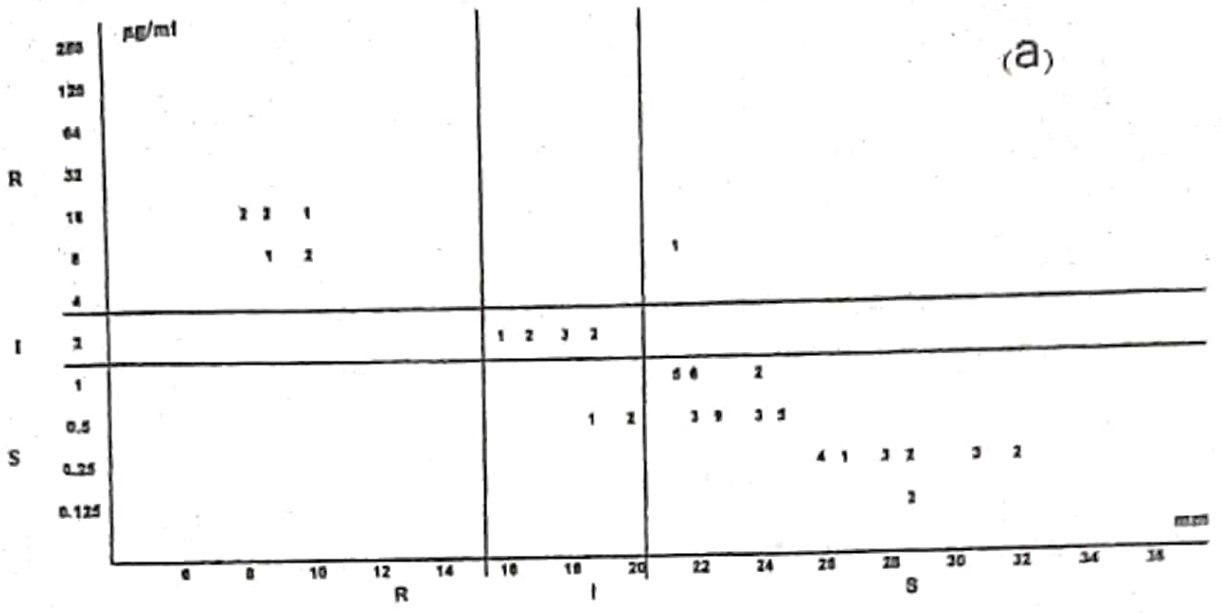
Ninety percent of *Staphylococcus* strains are resistant to penicillin and penicillin-derived antibiotics. The next line of attack, methicillin, is increasingly becoming less effective and the prevalence of methicillin-resistant strains of *S. aureus* has increased ~26%[15]. Recently, methicillin and multidrug-resistant *S. aureus* clones caused life-threatening infections in British and Uruguay[16, 17]. While non-hospital acquired *Staphylococcus* infections can be treated with penicillin-derived antibiotics, hospital-acquired infections are entirely resistant to penicillin and require more effective antibiotic treatments. *S. aureus* is one of the major causes of hospital-acquired infection and ranked fourth in a listing of the "Pathogens Most Frequently Isolated Form Hospitalized Patients[18]. Approximately 40% of the general population and 50–90% of health care practitioners harbor an *S. aureus* in their anterior nasal passage[19].

The antimicrobial sensitivity testing of the staphylococcal isolates revealed that the most effective drug is ciprofloxacin (77%) and the less effective drugs are ampicillin/ sulbactam (57%), gentamicin (55%) and chloramphenicol (50%). These results are consistent with the results of Schlegelova et al.[20] who found that high percent of staphylococci were resistant to β–lactam antibiotics. Moreover, staphylococcal isolates showed 91.6% susceptibility to ciprofloxacin, 91.5% to erythromycin, 87% to tetracycline and 99.3% to rifampicin[21]. Ciprofloxacin showed excellent activity against *S. aureus*[22] Ciprofloxacin exhibited moderate to low activity (5-13% non-susceptibility) against nosocomial *S. aureus* strains. High rate of non-susceptibility of *S. aureus* were found to gentamicin, tetracycline, erythromycin and chloramphenicol[23]. The susceptibility of stahphylococcal isolates to third generation cephalosporins ranged from 87-100% but there is increase resistance with time[24]. Dixon et al.[25] studied twenty clinical *S. aureus* isolates and found that the isolates were resistant to gentamicin and methicillin,, while amikacin was the most active where the isolates were inhibited by less than 1 µg/ml.

Table 7: Analysis of *S. aureus* isolates with discrepancies in comparison with E-test.

Antibiotic	Isolate no.	Disk diameter mm	Microdilution µg/ml	E-test µg/ml
CIP	4	10 (R)	0.5 (S)	0.25 (S)
	17	21 (S)	16 (R)	(32) R
	25	20 (I)	0.5 (S)	0.5 (S)
	33	18 (I)	0.5 (S)	0.5 (S)
CTX	7	12 (R)	1 (I)	0.75 (I)
	19	12 (R)	1 (I)	1 (I)
	24	24 (S)	8 (R)	32 (R)
	32	9 (R)	1 (I)	0.75 (I)
C	15	16 (I)	8 (S)	8 (S)
	17	15 (I)	8 (S)	4 (S)
E	3	19 (S)	2 (I)	1.5 (I)
	14	16 (I)	0.25 (S)	0 38 (S)
	21	15 (I)	0.25 (S)	0.25 (S)
	32	19 (S)	2 (I)	2 (I)
GN	2	15 (S)	8 (I)	8 (I)
	9	13 (I)	4 (S)	3 (S)
	15	15 (S)	8 (I)	8 (I)
	23	13 (I)	4 (S)	4 (S)
	32	14 (I)	4 (S)	4 (S)
RD	2	18 (I)	16 (R)	12 (R)
	9	R	12 (S)	1 (S)
	32	12 (R)	1 (S)	0.5 (S)
TC	4	14 (I)	2 (S)	2 (S)
	24	13 (I)	2 (S)	1.5 (S)

The obtained MIC for isolates in this study showed most MICs within serial 4 dilutions except for gentamicin which has a wider range. Similar results revealed wide ranges of MIC of several antibiotics against *S. aureus*. Ciprofloxacin and cefotaxime showed MIC_{90} of about 1 and 128 µg/ ml, respectively. Moreover, they noticed that oxytetracycline MICs ranged from 32-512 µg/ml and for neomycin, it was not more than 8 µg/ ml[26].


µg/ml
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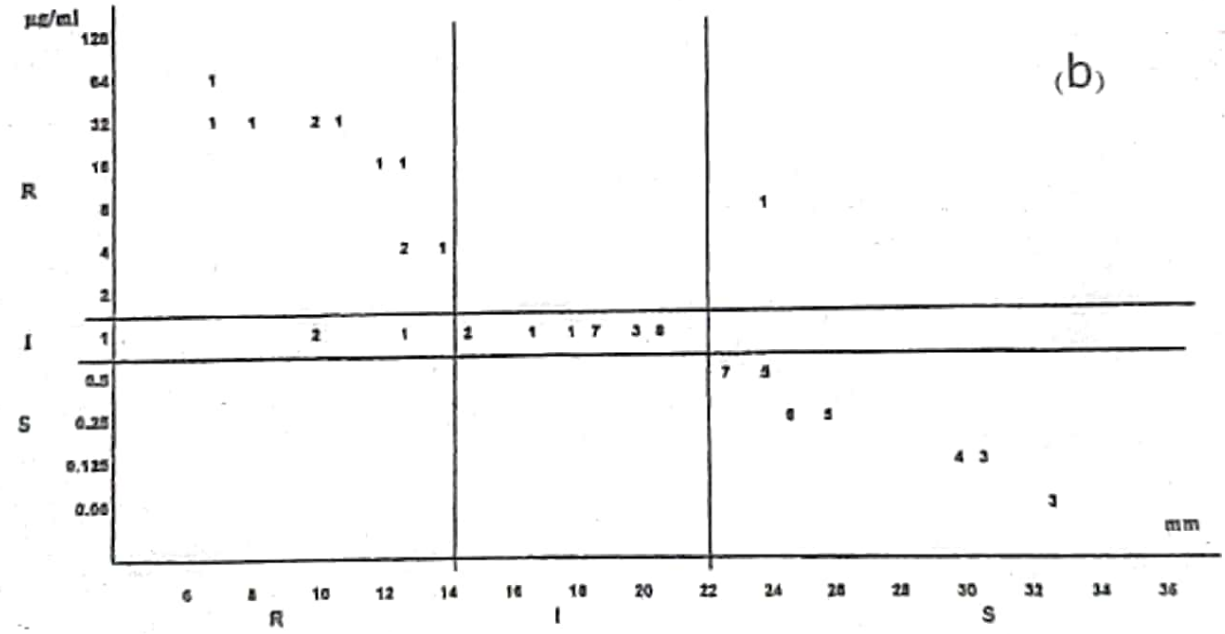
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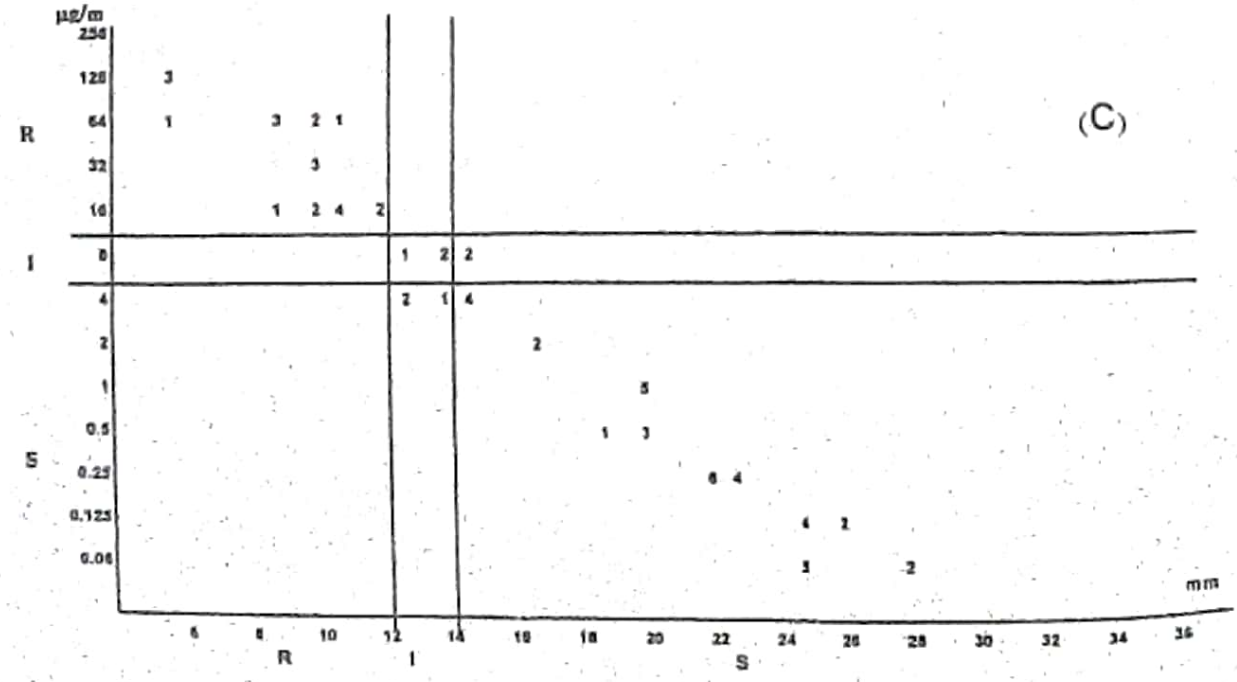
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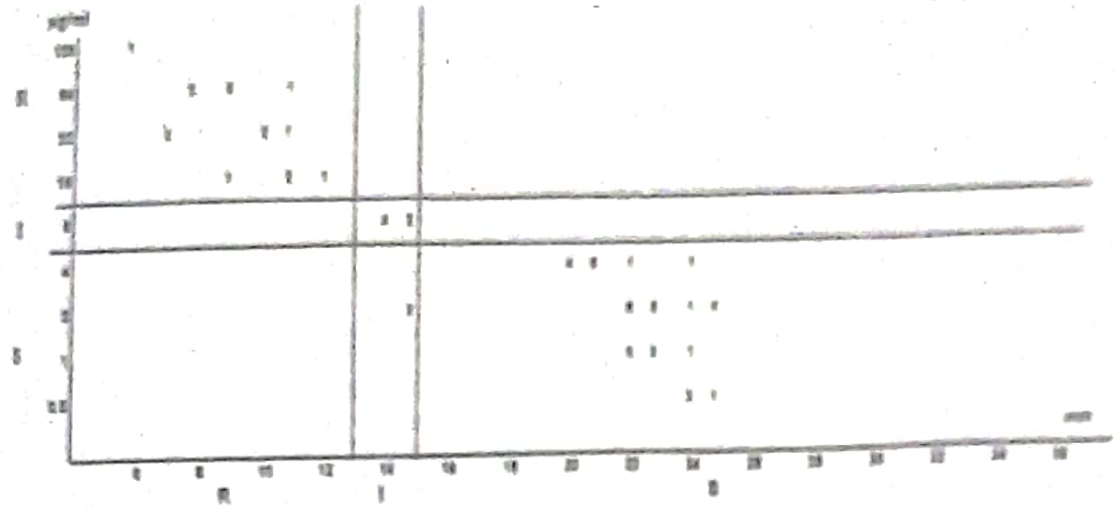

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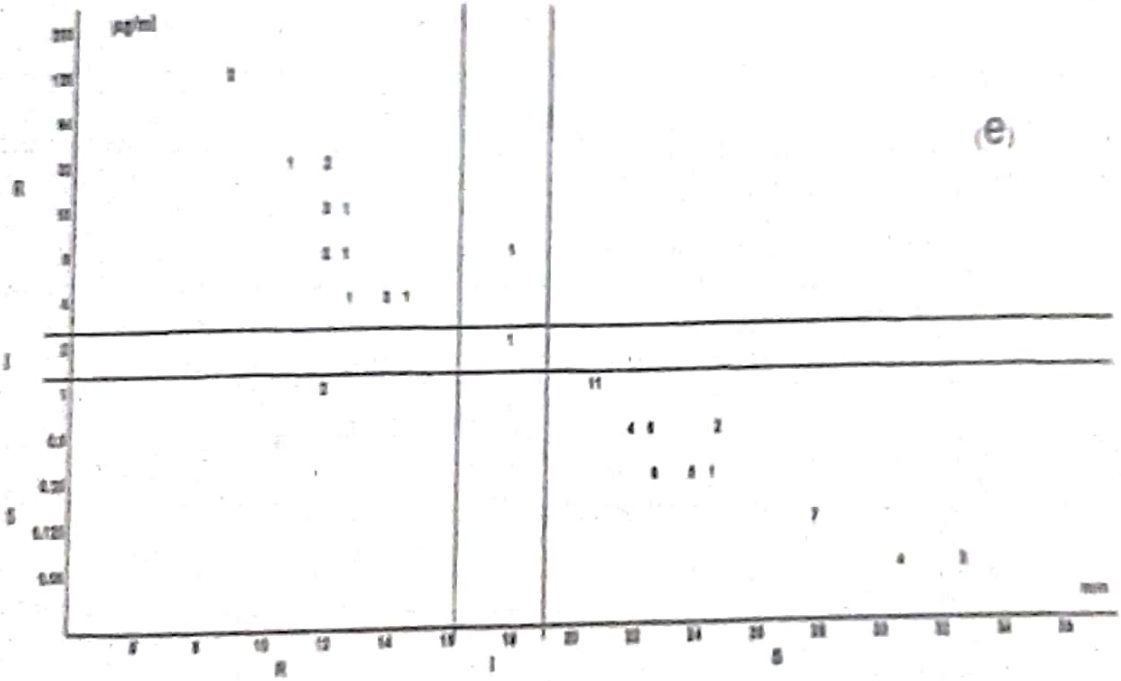
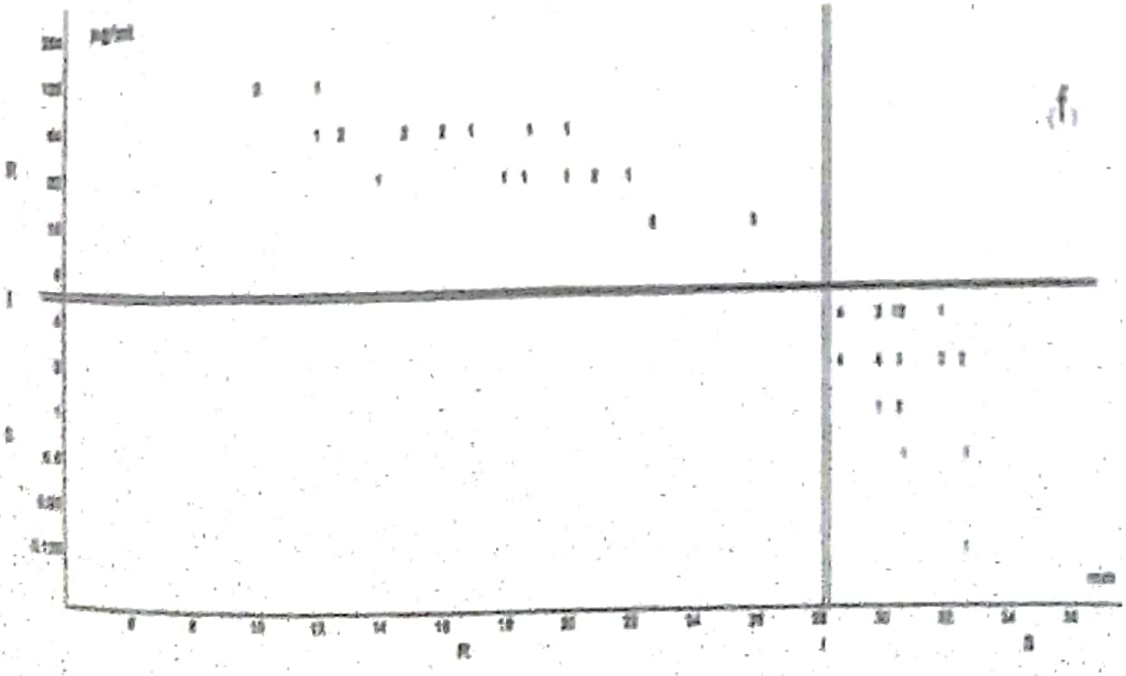


(f)



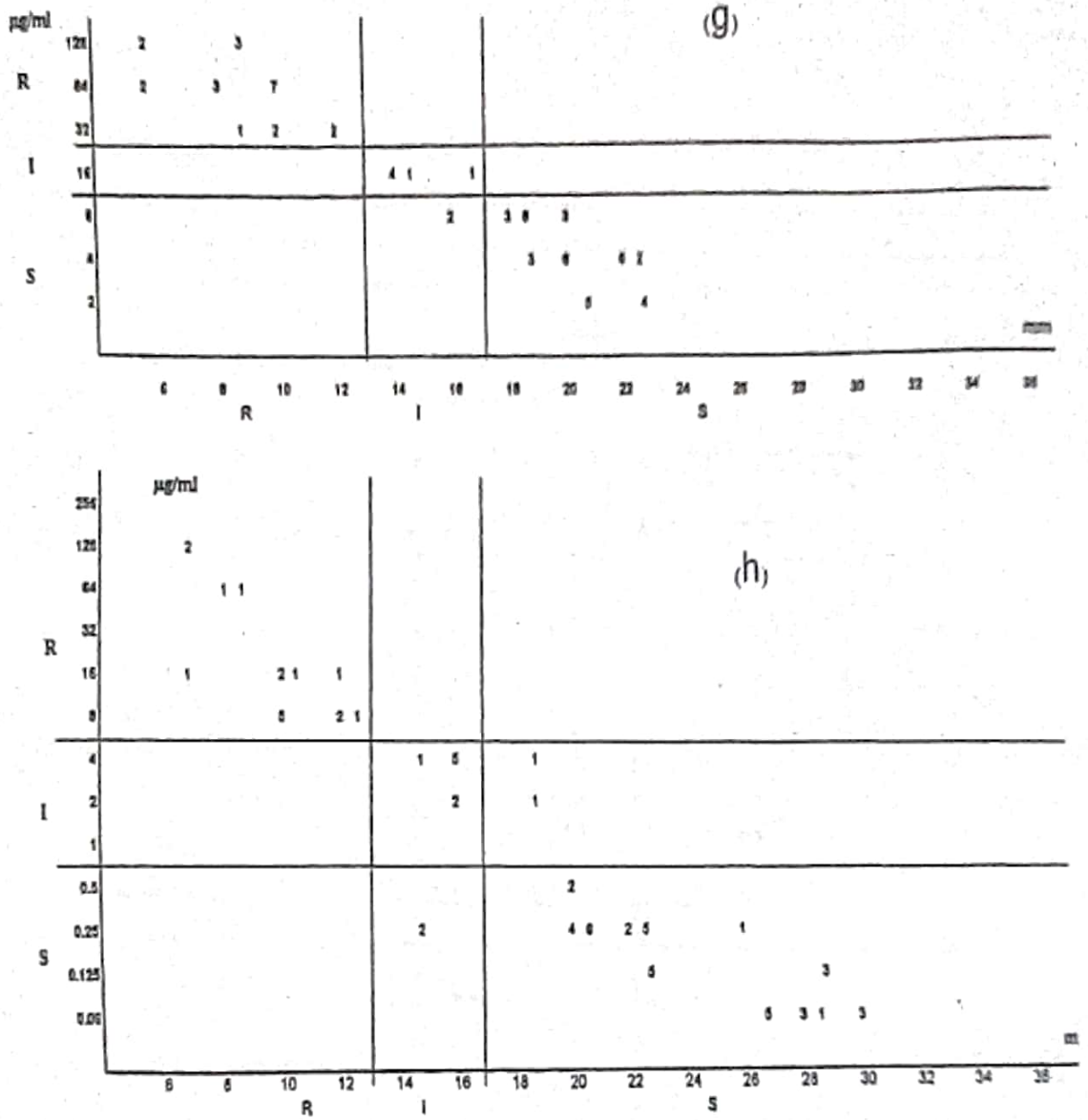


Fig. 1: Scattergram compare the MICs by broth microdilution to zone diameters around disks for 70 *S. aureus* isolates against ciprofloxacin (a), cefotaxime (b), chloramphenicol (c), erythromycin (d), gentamicin (e), rifampicin (f), ampicillin/ sulbactam (g) and tetracycline (h).

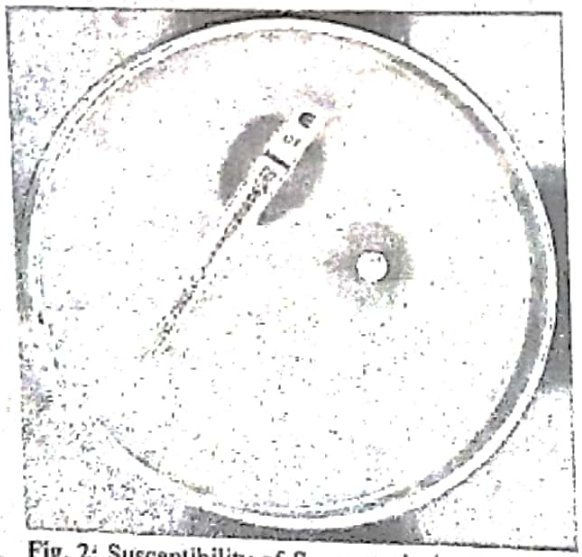


Fig. 2: Susceptibility of *S. aureus* isolate no. 24 tested against cefotaxime (CTX) by disk diffusion and MIC determination by E-test (CT). The strain was sensitive by disk diffusion (24 mm) but resistance in E-test (MIC= 32 µg/ml) according to NCCLS.

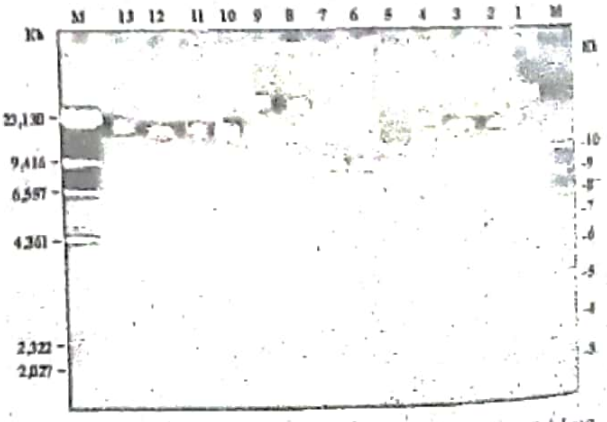


Fig. 3: Characterized plasmids DNA from multidrug resistant *S. aureus* no. 2, 3, 4, 7, 9, 14, 15, 17, 19, 23, 24, 25 and 23 (lanes 1-13), in respective manner. Lanes M are DNA fragment sizes marker, characterized plasmids with determined sizes (right side, Promega, California, USA) and λDNA digested with *EcoR*I and *Hind*III (left side, Böhringer, Mannheim, Germany).

Correlating the susceptibility as determined by broth microdilution and disk diffusion methods for tested isolates of *S. aureus*, high agreement was obtained (100%) with ampicillin/ sulbactam but lesser agreements (93%) were found with ciprofloxacin, gentamicin and chloramphenicol. Furthermore, very major discrepancy was detected with ciprofloxacin. While major discrepancy in gentamicin and minor discrepancy were found with other tested antibiotics except ampicillin/ sulbactam. Good relationship was obtained when MICs deduced from zone diameters of disk method compared with broth microdilution method for 7 antibiotics against 110 Gram-positive and Gram-negative bacteria[27]. Another study, detected very major and major discrepancies by 1% and minor discrepancy by 5-10% between MICs categories and disk results with enteric bacilli[28]. However, close results of very major, major and minor discrepancies were obtained with several antibiotics against *Streptococcus pneumoniae*[8]. It was noted that E-test MICs compared with conventional methods were always under estimation ±1- 2 dilutions[29]. There were a high degree of agreement between MICs of both E-test and microdiltuion method; 90% agreement and 100% sensitivity[30] and 95.1% essential agreement[8, 31].

Acceptable correlation was obtained in this study by comparison the results of disk diffusion and E-test. Trolldenier et al[32] detected satisfactory agreement between the two methods and in addition, there were misclassification in the agar diffusion test when examining streptococcal strains against 4 β–lactam antibiotics. Excellent correlation was also found between E-test, broth microdilution and disk diffusion test in the results of mupirocin resistant and susceptible staphylococcal strains[18]. However, E-test results are the most accurate, reliable and the nearest to the reference broth microdilution results and more than disk diffusion method. Moreover, E-test is recommended as the best and simplest method for routine antibiotic sensitivity when examining metronidazole against *H. pylori*[33] and glycopeptides against *S aureus*[34] and 8 antibiotics against *Pseudomonas areuginosa*[35]. Huang et al[36] compared also the results of E-test MICs with that of agar dilution technique for 18 antibiotics against several bacterial isolates, including staphylococci, *C. jejuni* and multidrug resistant enterococci. They reported that the overall agreement of MICs was 97% for staphylococci, 82% for campylobacter and 100% for enterococci. The accuracy of E-test was 90.4% with 100% reproducibility. The MICs values ranged to be ± 1.0 log 2 dilutions when E-test results compared with agar dilution results for sparfloxacin, ceftazidime, cefprozil, cefdinir, aztreonam, tobramycin and amikacin. The major error was rare and represented 0.1% of test strains[37]. In Norway, *S. aureus* clinical strains showed MIC of ≥2 µg/ml for bacitracin, ≤ 0.5 µg/ ml for mupirocin and about 91% strains were with MIC of ≥16 µg/ ml[38].

Recently, NCCLS has three categories 1) susceptible means infecting organism is usually inhibited by concentration of a particular antibiotic attained in tissues by usual dosage, 2) intermediately susceptible where the infecting organism is inhibited by blood or tissues concentration achieved by maximum dosage, 3) resistant where the organism is resistant to normally achievable and tolerated concentrations of antimicrobial drugs. Multidrug-resistant staphylococcal isolates reached about 94% was obtained in this study. Similar results were obtained of about 94 and 93% of staphylococcal isolates resistant to one and two or more antibiotics, respectively[20]. In Lebanon, multidrug-resistant *S aureus* clones were found with 96, 44, 34, 29, 20, 10, 7 and 3% resistance to penicillin G, tetracycline, amikacin, augmentin, sulfmethoxazole-trimethoprim, chloramphenicol, erythromycin and gentamicin and tobramycin, in respective manner[39].

Clinical isolates of *S. aureus* commonly possesses one or more plasmids on which antimicrobial resistance determinants are frequently encoded. The plasmid range from small rolling-circle (RC) plasmids that carry a single resistance determinant and are multicopy to large multi-resistance and conjugative plasmids that are generally 15-60 kb in sizes and maintained at low copy number[40]. Plasmids of 8-30 kb in their sizes were detected and isolated from clinical *S. aureus* isolates in this study with the transfer of ampicillin, cefotaxime, chloramphenicol and tetracycline resistance to *E. coli* competent cells. The plasmids might be conjugative and originating from human and animal sources. Plasmids of sizes ranged 2.224-20.650 kb were extracted and purified from *S. aureus* clinical isolates resistant to antibiotics and metals[41]. Another two small plasmids of 2.910 and 2.889 kb were characterized with chloramphenicol resistance determinant in nosocomial mltidrug-resistant *S. aureus*[42]. Similarly, a large plasmid of 25.9 kb with metal cadmium resistance was isolated from *S. aureus*[43]. Huys et al.[44] reported that tetracycline resistance in *S. aureus* is mainly disseminated by transmissible plasmid such as pT181 or by conjugative transposons such as Tn*916*. However, broad-host conjugative plasmids of 45 and 95 kb were detected and purified from *Enterococcus faecalis* and *S. aureus* with resistance to vancomycin, erythromycin, streptomycin and gentamicin[45]. Moreover, Tn*1546*-like elements of *Enterococcus*

faecalis origin were cloned in vancomycin-resistant *S. aureus*[46]. Furthermore, a plasmid of 46.4 kb was cloned from vast majority antibiotic resistant clinical strains of *S. aureus* and considered it is the prototype of conjugative staphylococcal multi-resistance plasmids family[47]. This means that the plasmids and transposons are horizontally inter and intraspecies transferred with the broad transfer of antimicrobial agents resistances.

Collectively, treatment of *S. aureus* infections showed no signs of broad-antibiotic resistance and could be treated with one or two of the following antibiotics: ciprofloxacin, cefotaxime, ampicillin/ sulbactam and rifampicin. Plasmids and transposons are the principal genetic elements that responsible for the horizontally transfer of antibiotic resistance in *S. aureus*. E-test is considered the best and simplest method for routine antibiotic sensitivity and rapid determination of MICs,

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إختبار حساسية عزلات إكلينيكية من الميكروب الذهبى العنقودى بطرق مختلفة ودراسة مقاومتها للمضادات الحيوية

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لقد تم تصنيف عدد ٧٥ عزلة من الميكروب المكور العنقودى منها ٧٠ عزلة مكور عنقودى ذهبى موجبة لإختبار التجلط. وباستخدام طريقة الإنتشار بالقرص (Disk diffusion) لقياس درجة الحساسية إتضح أن السبروفلوكساسين هو الأكثر فاعلية (٧٧%) وأن الكلورمفينيكول هو الأقل فاعلية (٥٠%). وباستعمال طريقتى التخفيف المتسلسل الحسائى الدقيق و التخفيف الآجارى (Broth microdilution and agar dilution methods) لتحديد التركيز الأدنى المثبط (MIC) والتركيز اللازم لوقف نمو ٥٠% (MIC_{50}) و ٩٠% (MIC_{90}) ولحساب درجة المغايرة والإختلاف بين النتائج (Discrepancy)، فلقد وجد توافق بمعدل ١٠٠% بين العزلات والمضاد الحيوى أمبسللين/ سيلبكتام وكانت أقل نسبة توافق قد وجدت فى المضاد الحيوى جنتاميسين (٩٣%).

وباستخدام أختبار- هـ الذى يعتبر حالياً إختباراً كمياً ونوعياً لتحديد التركيز الأدنى المثبط للمضادات الحيوية، قد أتضح من النتائج أن هناك توافق عالى بين نتائج إختبار- هـ ونتائج طريقتى التخفيف المتسلسل الحسائى الدقيق و التخفيف الآجارى بنسبة ١٠٠% لأغلب العزلات ولكن قد يكون هناك إختلاف فى التركيز الأدنى المثبط بفارق تخفيف أو إثنين أعلى أو أقل بينهم.

وبفصل وتنقية البلازميدات من العزلات الأكثر مقاومة للمضادات الحيوية، وجد أن البلازميدات الموجودة يتراوح حجمها بين ٨-٣٠ كيلو قاعدة نيتروجينية. وبنقل هذة البلازميدات إلى خلايا الإشيريشيا كولاى المكافئة، وجد أن خلايا الإشيريشيا كولاى قد إكتسبت مقاومة للمضادات الحيوية أمبسيللين، سيفوتاكسيم، كلورامفينيكول والتتراسيكللين. ولهذا أعتبرت البلازميدات هى المسئولة جينياً عن نقل وأنتشار المقاومة للمضادات الحيوية بين خلايا عزلات المكور العنقودى الذهبى محل الدراسة.

بعد إجراء هذا البحث إتضح أن أختبار حساسية الميكروبات باستخدام طريقة الانتشار بالقرص خلال الآجار تعطى نتائج يمكن الاعتماد عليها نوعياً وليس كمياً بينما لتحديد التركيز الأدنى المثبط لنمو الميكروبات يمكن تحديده بطريقة إختبار- هـ الحديث بغض النظر عن التكلفة الاقتصادية حيث يعد الطريقة الأمثل والأسرع.