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Patterns of Antibiotic Resistance in *Staphylococcus aureus* Isolates and Detection the Heteroresistance to Vancomycin by Population analysis Method

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A R T I C L E   I N F O

A B S T R A C T

Staphylococcus aureus is a major cause of bacterial poisoning and spread widely in Iraq. In this study vancomycin resistant *S. aureus* (VRSA) were isolated from wounds, skin, and nose of human . The isolates were identified by using biochemical tests.Sixty one (72.6%) isolates were identified as *S.aureus*, followed by CoNS 23 (27.3%) from 250 sample collected. Antibiotic susceptibility was determined by disk diffusion ,the results of the susceptibility test indicated that 59 *S. aureus* isolates have different levels of resistance to antibiotics.In this study tow methods were used to identify resistant and intermediate resistance to vancomycin: which were Kirby-Bauer disk diffusion and automated system Vitek2 method. Results of Disk diffusion method indicated that (19.6%) isolates were resistant to vancomycin.The results of Vitek2 resistant test for 20 isolates indicated that 9(45%) isolates were resistant to vancomycin, with MIC value of (32 μg / ml); 3(15%) isolates showed intermediate resistant to vancomycin, with MIC value of (4 μg / ml),8(40%) isolates showed sensitive to vancomycin with MIC value of (≤0.5-2 μg / ml).Population analysis profile (PAP) method was uesd to detect Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* for the 10 isolates. The results showed that 9 (90%) isolates of *S.aureus* were resistant to vancomycin , while 1(10%) isolate was sensitive.

Introduction

Staphylococcus aureus is one of the commonest causes of healthcare-associated bacteremia [1] and a major human pathogen that causes a wide range of clinical infections. It is a leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, bone infections, nosocomial infections, surgical wound infections and device-related infections [2].

Several virulence factors are implicated in the pathogenesis of *S. aureus* strains have been described. These include surface components (capsule, peptidoglycans, teichoic acid, protein A, cell attachment protein), enzymes (coagulase, lipases, esterases, proteases, hyaluronidase, deoxyribonuclease*,* catalase, beta-lactamase, and staphylokinase), and several toxins [3].

Using of vancomycin has increased dramatically worldwide as a result of empirical and directed therapy against burgeoning MRSA infections. VRSA strains have been isolated from USA, France, Korea, South Africa, Brazil, Japan, and Scotland. Extensive use of vancomycin creates a selective pressure that favors the outgrowth of rare, vancomycin-resistant clones leading to heterogeneous vancomycin intermediate *S. aureus* clones, and eventually, with continued exposure to a uniform population of VISA clones [4,5].

Due to the importance of *S. aureus* both as humans and animals pathogen this study was carried out with the several objectives . To detect and

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characterize *Staphylococcus* isolates from human sources using different biochemical tests . Based on the results, possible VISA and VRSA strains will be identified as homogeneously or heterogeneously vancomycin resistant by performing population analysis . The results will be confirmed by using antibiotic susceptibility test (Vitek2).

Materials and Methods:

Samples collection:

During the period from February to May 2016, 250 samples (120 nasal and 130 skin samples) were collected from Al-Nu'man General Hospital, Al-Yarmouk Teaching Hospital, and Abu Ghraib General Hospital in Baghdad and Baghdad medical city teaching hospital and then transported directly to the laboratory at Al-Anbar University. The samples were cultured on blood agar; and mannitol salt agar that is used for selective isolation (contain 7.5 % NaCl), and for culturing and differentiating of medically important Staphylococci species.

Isolation and Identification of Staphylococci:

The isolation of staphylococci from clinical samples were carried by specific way depending on routine laboratory techniques, all samples were streaked on mannitol salt agar and all plates were incubated aerobically for 24 hrs. at 37°C. *S.aureus* isolates were identified depending on the morphological features on culture media and biochemical tests according to Bergey's Manual [6].

Susceptibility to antibiotics and Determination of Minimal Inhibitory Concentration (MIC):

Antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton agar based on Clinical and Laboratory Standards Institute guidelines [7]. Susceptibility test was determined for *S. aureus* isolates   against 14 different antibiotics used: Vancomycin, Amoxicillin, Amoxicillin(Clavulinic acid), Azithromycin, Cefoxitin, Clindamycin, Erythromycin, Gentamycin, Levoflaxacin, Methicillin, Penicillin, Rifampin, Sparfloxacin, and Tetracycline.

The minimal concentration of vancomycin that inhibit the growth of *S.aureus* was determined by vitek 2 compact system.

Population Analysis

Population analysis of Glycopeptides Intermediate *S. aureus* (GISA) and hetero Glycopeptides Intermediate *S. aureus* (hGISA) isolates was performed according to the method of Hiramatsu [8]. Isolates were suspended in brain-heart infusion broth to a 2 McFarland standard (6×10^8 CFu/mL). Inoculums of 3×10^7 CFu were placed on brain-heart infusion agar plates containing serial dilution of vancomysin (1-10mg/L). Plates were incubated at 37°C for 48h and colonies were counted.

Results and Discussion

S. aureus produced yellow colonies with yellow zone on mannitol salt agar as a result of utilizing mannitol (positive result for mannitol fermentation). The other species of Staphylococci produced small pink or large deep yellow to deep orange colonies with no color change of the medium [9].

The isolated colonies were purified by ABC streaking method on mannitol salt agar; the isolates were then examined microscopically for gram stain, shape, and cluster arrangement. Eighty-four isolates were identified morphologically as gram positive cocci, arranged in grape-like irregular clusters .The clusters occurred because the bacterial cells divided to three planes in an irregular pattern producing branches that considered as characteristics of *Staphylococcus spp*. [9,10].

For further identification, the catalase test was preformed for the 84 isolates that gave positive results with catalase test, and this differentiates *Staphylococcus* from the genus *Streptococcus* which gave negative results . The oxidase test was also applied for the 84 isolates; negative results were observed with all 84 isolates that differentiate *Staphylococcus* from the genus *Micrococcus* which usually produces purple color as positive result [9,11,12].

After identification of the isolates at generic level, the coagulase test was performed to identify the bacterial isolates, sixty one isolates (72.6%) reveled the ability to produce coagulase enzyme (coagulase positive), and twenty three isolates (27.4%) were coagulase negative.

For further identification, Baird-Parker medium was used. In this study all 61 *S. aurues* isolates grown in this medium (Figure 1). This medium is a modification of a previous formula developed by Zebovitz *[13]*

Figure 1. *Staphylococcus aureus* **isolates on Baird-Parker medium**

The results indicated that out of 84 isolates, 61 (72.6 %) were identified as *S.aureus* and 23 (27.3 %) were identified as Coagulase Negative *Staphylococcus* (CoNs), Vitek2 was employed for the result confirmation, the card for detection of gram positive bacteria. Regarding the source of samples and the isolated *Staphylococcus* spp., the results appeared as follows: out of 120 nasal swabs, 30 was CoPS and 13 was CoNS; for the 130 burn swabs, 31 was CoPS, and 10 was CoNS as showen in table (1) .

The results agreed with [14,15,16] who indicated that nasal cavity is considered the major reservoir for staphylococci.

Table 1. Isolated *staphylococcal* spp. from clinical sample

Sample Source	Sample No.	CoPs	CoNs
Nasal swabs	**120**	**30(25%)**	**13(10.8%)**
Burn swabs	**130**	**31(23.8%)**	**10(7.6%)**
Total	**250 (100%)**	**61 (24.4%)**	**23 (9.2%)**

Antibiotic susceptibility

Staphylococcus aureus susceptibility test was done for the 61 isolates of S.aureus using disk diffusion method; the test was applied for 14 different antibiotics. Results of the susceptibility test indicated that 59 S. *aureus isolates* have different levels of resistance to antibiotics*:* Methicillin (89.8%), Penicillin (87.8%), Cefoxitin (77.1%)*,* Amoxicillin (51.6%),   Amoxicillin Clavulinic acid (52.5%), Vancomycin (27.1), Erythromycin (95.6%), Clindamycin (96%)*,* Azitheromycin (74.1%), Gentamycin (58.3%), Rifampin (58%), Tetracycline (8.2%), Levofloxaciin (3.5%), and Sparfloxacin (0%). figures (2) .
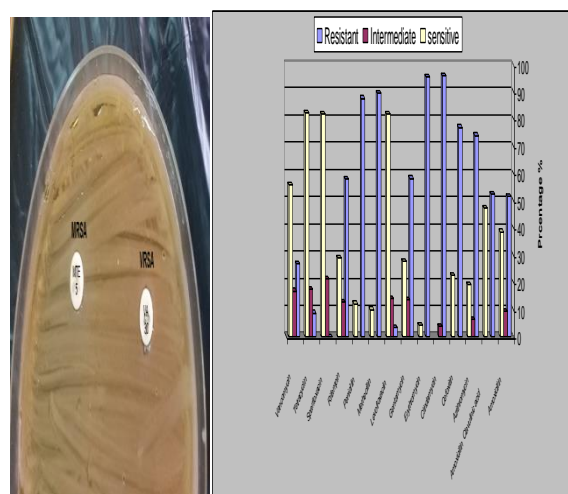
a   b
Figure 2.   *a*- Methicillin and Vancomycin susceptibility test by disk diffusion method
b- Antibiotic susceptibility of *Staphylococcus aureus* isolates

In this study, the prevalence of Methecillin Resistance *S.aureus* MRSA was (89.8%), which varied from findings of other studies in other countries. In three separate studies in Iran, 56%, 72%, and 58% of staphylococci isolates were identified as methicillin resistan [17,18,19]. Al-Hasani (2011) showed that Methicillin resistant to *S. aureus* was (83.7%).

Whereas [20] indicated that penicillin resistant was (89.2%) for *S. aureus* isolated from pus and wound swabs.

The difference in rates of isolation of MRSA in different studies might be due to the difference in locations and time periods of the studies, and difference in hygienic conditions maintained in different hospitals [21], healthcare facilities provided by the hospital, implementation of infection control program, and rational use of antibiotics, which may vary from hospital to hospital [22].

It is quite possible that the Amoxicillin-Clavulanic acid complex (52.5%), which is a larger molecule than Amoxicillin (51.6%), may experience greater difficulty in permeability and overall transport across the microbial cell wall membrane barrier.

In this study, 77.1% of S. *aureus* isolates exhibited Cefoxitin resistance by disc diffusion method; previous studies mentioned 69.1% and 68% resistance [23,24] ,respectively . Gentamicin resistance was (58.3%) among isolates; [25] found that Gentamycin resistance was (52.5%) . The tetracycline resistance was (8.82%) among *S. aureus* isolates. [25] found that Tetracycline resistance was (70%), while [26] showed that Tetracycline resistance was (55%).

[27] showed tetracycline resistance rates by years (10.1%) among *S. aureus* isolates.

The result of *S. aureus* resistance to Rifampin was (58%) in this study. [28] indicated (94.3%) resistance to Rifampin was, while (17.3%) of isolates showed resistance to Rifampin [29]. The causes of Rifampin-resistant mutations within bacteria might be due to alterations in the gene, which encodes the β-subunit of the RNA polymerase enzyme [30].

The results of resistance *S. aureus* isolates were (0%, 3.5%) for Sparfloxacin and Levofloxaciin, respectively; with 82.1% for Sparfloxacin and Levofloxaciin susceptibility. [20] showed that Sparfloxacin susceptibility was (75.6%) and Levofloxaciin susceptibility was (69.6%).

The results showed 95.6%, 96%, and 74.1% resistant to Erythromycin, Clindamycin, and Azithromycin, respectively. [31] mentioned that Clindamycin resistance was found in 10% of *S. aureus* isolates, while [27]found 13.5% resistance among *S. aureus* isolates. [32] revealed that the percentage of Erythromycin resistance among *S. aureus* isolates was (28.42%).While [33] showed that Erythromycin resistance among *S. aureus* isolates was (85.7%) and was (78%) for Azithromycin.

Vancomycin sensitivity test results indicated the existing of 61 isolates of *S. aureus*: 12 isolates (19.6%) were resistant to Vancomycin, 8 isolates (13.1%) were intermediate resistance, and 41 isolates (45.9%) were sensitive. [34] findings stated that vancomycin resistant *S. aureus* were 30% among isolates. [35] and [36]showed that VRSA were 20% and 41.2% among *S. aureus*, respectively. Also, rate of resistant to vancomycin among *S. aureus* isolates was (2.27%) according to [37], (8%) according to [38], and 2.8% according to [39].

In this study, MRSA represented 89.8%, this result demonstrated high prevalence, and increased distribution of Methicillin resistant Staphylococci isolates in the community among carrier persons and patients; This is in agreement with other studies [40,41] . The prevalence of VRSA among MRSA was 93.75%, while prevalence of VISA and VSSA was 90% and 87.87%, respectively.

Determination of Minimal Inhibitory Concentration (MIC)

The MIC of Vancomycin for *S. aureus* isolates was determined by Vitek2. The results showed that VRSA isolates had MIC (≥32 *μg/ml*) and VISA isolates had MIC (4 μg/ml), while VSSA isolates had MIC value ranged from (≤0.5-2 *μg/ml*) .

The results of antibiotics susceptibility by Vitek2 indicated that 20 isolates (100%) were MLSB+SA resistant, 16 isolates (80%) were Beta lactamase resistant, 7 isolates (35%) were Glycopeptides resistant, and 5 isolates (25%) were Oxazolidinone resistant , as shown in table (2).

Table 2. Patterns of Antibiotic Resistance in *Staphylococcus aureus* Isolates

Antibiotic Resistance Patterns	MLSB+SA resistant	Beta lactamase resistant	Glycopeptides resistant	Oxazolidinone resistant
S.aureus isolates No. (%)	20(100%)	16(80%)	7(35%)	5(25%)

The multidrug resistance (MDR) bacteria defined as resistance to 3 or more types of antibiotics [42]. Multidrug resistant bacteria including *S. aureus* can be isolated from different infection [43]. In the present study, VRSA showed resistance to a wide range of antimicrobial agents. All VRSE and VISE isolates showed multidrug resistance; even VSSE exhibited multidrug resistance.

Disk diffusion test appears not accurate for determining vancomycin susceptibility; broth dilution or the E-test method should be used instated [44]. E-test demonstrated to read slightly higher than broth microdilution (BMD) Method, it may be the best alternative for evaluating MRSA vancomycin MICs in patients with serious and life-threatening infections [45]. [46] showed that E-test gave an MIC value greater than that of Vitek2 in 64(85.3%) isolates.

In this study, comparison was made between the two methods used for measuring the sensitivity of the isolates to vancomycin. Differences emerged especially between disk diffusion and Vitek2; 12 isolates were VRSA by disk diffusion, and 9 isolates were VRSA by VITEK 2 as shown in table (3). VISA and VRSA isolates were not detected precisely by the disk diffusion method; the acceptable methods used to detect these isolates were non-automated and include broth or agar dilution and the E-test method [47].
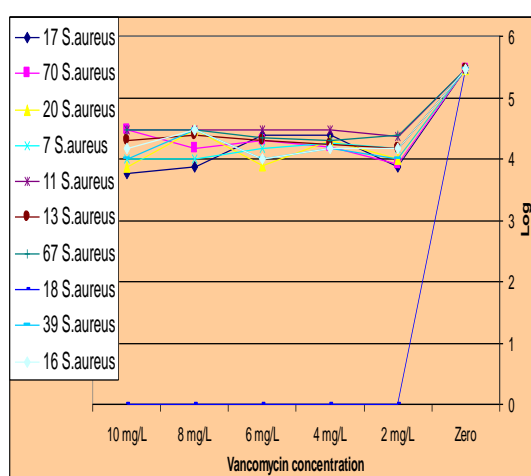
Population analysis

Results of vancomycin susceptibility obtained by Vitek2 showed that 3 isolates were VISA, 9 isolates VRSA, and 8 isolates VSSA. The population analysis profile showed that 9 isolates were resistant to vancomycin and one isolates was sensitive to vancomycin as presented in figure (3).

Table 3. Comparison among vancomycin resistant phenotypes of 20 isolates *S. aureus* by two methods

Methods	VRSA	VISA	VSSA
Disk diffusion	12 (60%)	8 (40%)	0
Vitek2	9 (45%)	3 (15%)	8(40%)

Figure 3. Population analysis profile curves for vancomycin-resistant and sensitive for vancomycin *S. aureus* isolates

The emergence of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) over the past decade has provided a challenge to diagnostic microbiologists to detect these strains, clinicians treating patients with infections due to these strains, and researchers attempting to understand the resistance mechanisms. By using population analysis profile (PAP) as a reference method, the hVISA phenotype can be detected for strains of *S. aureus* with vancomycin MICs as low as 0.5 to 1 µg per ml [48]. In a clinical study, the hVISA phenotype was detected in 50% of clinical MRSA isolates with vancomycin broth MIC of 2 µg per ml [49]. In this study,the 9 VRSA isolates were highly resistant and homogeneously.

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أنماط مقاومة المضادات الحياتية في عزلات المكورات العنقودية الذهبية *Staphylococcus aureus* والتحري عن تغاير مقاومة الفانكومايسين باستخدام طريقة population analysis

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الخلاصة:

بكتيريا المكورات العنقودية الذهبية هي السبب الرئيسي للتسمم البكتيري وقد انتشرت على نطاق واسع في العراق. تم عزل هذه البكتيريا المقاومة للفانكوميسين من الجروح والجلد والأنف من الانسان. وشخصت باستعمال الاختبارات الكيموحيوية. تم عزل 61 (72.6%) عزلة من المكورات العنقودية الذهبية *S.aureus*، و23(27.3%) عزلة من النوع المكورات العنقودية السالبة لانزيم التخثط (CoNS). حددت الحساسية للمضادات الحياتية باستخدام طريقة الانتشار على الاطباق (disk diffusion), اشارت نتائج الاختبار ان 59 عزلة من *S.aureus* اظهرت مستويات مقاومة مختلفة للمضادات الحيوية . في هذه الدراسة، تم استخدام طريقتان لتحديد العزلات المقاومة والمتوسطة المقاومة للفانكومايسين وهي طريقة الانتشار على الاطباق (disk diffusion) والنظام الآلي Vitek2. أشارت نتائج طريقة الانتشار على الاطباق انه (19.6%) عزلة كانت مقاومة للفانكومايسين. أظهرت نتائج طريقة Vitek2 أن 9 (45%) عزلة من *S.aureus* كانت مقاومة للفانكومايسين حيث كانت قيمة (MIC) للعزلات (32≤) (µg/ml) و3(15%) عزلة من *S.aureus* ظهرت متوسطة المقاومة للفانكومايسين حيث كانت قيمة (MIC) للعزلات (4 µg/ml) و8 (40٪) عزلة ظهرت حساسة للفانكومايسين حيث كانت قيمة (MIC) للعزلات (0.5-2≥)(µg/ml).استخدمت طريقة Population analysis للكشف عن تغاير مقاومة عشر عزلات من المكورات العنقودية الذهبية للفانكومايسين . أظهرت النتائج أن 9(90%) عزلة من *S.aureus* مقاومة للفانكومايسين حسب المنحنى الخاص بالتجربة 1, (10%) عزلة من *S.aureus* حساسة للفانكومايسين.