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The Differences in Antibiotic-resistance among Several *Staphylococcus aureus* strains in Iraq

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Abstract

Background: The spread of antimicrobial-resistance had continuously emerged among *S. aureus* strains poses a major concern in treating the infection with this bacterium and established a challenge to clinical laboratories. Consequently, measuring resistance is essential to provide a clinical service for patients with *S. aureus* infections.

Methods*: S. aureus* was isolated from burn and wound injuries and identified according to the biochemical tests, then genotyped through *Spa*-typing method to diagnose at the strain level.The antibiotic resistance patterns of the 18 *S. aureus* strains were studied by Kirby-Bauer assay and the inhibition zone was measured.

Results: The antibiotic results of each *spa*-type were discussed according to the globally published researches. Moreover, the *spa*-type of this study was grouped into two groups, one with high resistance and the other with less resistance.

Conclusion: In conclusion, the high resistance bacterial group infects injuries that needed a long period to be healed, and the refore it is recommended to have a strict sterilized separated environment for cases most prone to infections such as patients with a burn or diabetic foot injuries.

Keywords*: Antibiotics, burn, diabetic foot, Staphylococcus aureus, spa-type, wound*

Introduction

S. aureus is the most invasive species and an etiological agent of diverse human and animal maladies, it is isolated from the community and can cause community-acquired infections and it is responsible for nosocomial infections when isolated in hospitals, it may affect the lower respiratory tract, urinary tract, skin and bloodstream [(1)].

The source of *S. aureus* infections can be (endogenous) from the patient's anterior nares or by transferring from other reservoirs (exogenous infections). Exogenous reservoirs can often be nasal carriage in the medical staff and transmitted by their hands or aerogenic transmission by binding to particles of dust then transmitted to susceptible sites. Moreover, contaminated instruments and devices have also been identified as transient reservoirs for the spread of *S. aureus*[(2)].

S. aureus can persist for a long time especially in inadequately cleaned areas due to its ability to survive the dry conditions also can survive on surfaces of skin scales for up to 80 days [(3)]. Some cases increases the risk of infection such as the hospitalized patients with general weakness and immune system suppression, particularly patients with burn injuries which they had lost their protective skin barrier and their immunological variation[(4, 5)].

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The emergence of bacteria with multiple antibiotic-resistant, which persist and spread worldwide, will compromise the treatment of infections causing clinical failures of these treatments[6]. Locally at burn units, several pathogenic bacteria, including *S. aureus*, were found with a high percentage of resistance to the commonly used antibiotics[7]. Antibiotic resistance is the most important characteristic of bacterial pathogenicity. The incidence of antibiotic resistance has commonly grown in several bacterial groups, including the staphylococci. The increase in resistance can be due to frequent antibiotic administration resulting in selective pressure on bacteria [8]. Also, the spreading of drug-resistance genes via effective vehicles called mobile genetic elements (MGEs) through horizontal gene transfer between *S. aureus* strains, will change the pathogen's abilities to cause the disease and has a significant impact on the organism's evolution [9].

It is of concern that the sensitivity of the wild-type bacteria to a specific antibiotic will not return after its absence, as in the case of rifampin-resistant *S. aureus*[10].

The examination of antibiotic-resistance according to *S. aureus spa*-types was not previously recorded in Iraq. In this study, *S. aureus* was genotyped by *spa*-typing method and the clinical cases with high resistance to the studied antibiotics were investigated.

Material and Methods

Bacterial Isolation and Identification

One hundred Burn, wound, and environmental samples had been swabbed then cultured on a selective medium (Mannitol salt agar) that differentiate *Staphylococcus aureus* through the fermentation of mannitol and formation of acidic products which reduce the pH of the medium, and this will turn the phenol red indicator to yellow [11]. The microscopic examination by Gram stain was performed for the isolates that ferment mannitol of Manitol salt agar medium. Only the Gram-positive grape-like clusters were preserved at Nutrient agar slants at the refrigerator (4℃) to complete the tests of *S. aureus* identification.

A biochemical test such as catalase test was then performed, to indicate catalase enzyme through adding one drop of (3%) H_2O_2 on a glass slide then adding bacterial colony on it, bubbles will then appear as a positive result [11]. Another biochemical test was performed to detect another Staphylococcal enzyme through tube coagulase test. By using human plasma at the dilution (1:5) with autoclaved Distal water (D.W) which will be added to an equal volume of cultured Nutrient broth medium.

Coagulase enzyme will clot plasma after 2 to 4 hours of incubation at 35℃ and sometimes the clot will appear after overnight incubation [12, 13].

Spa- typing of *S. aureus*

Bacterial DNA was isolated by using Wizard Genomic DNA Purification Kit/Promega. The concentration value of DNA was detected using Quantus Fluorometer by mixing 199 µl of Quanty Flour diluted dye with 1 µl of extracted DNA, then incubated at room temperature for 5 min, to investigate the goodness of samples and perform the downstream applications.

PCR amplification was done through preparing of the primers in final concentration (100pmol/µl)as a stock solution by adding nuclease-free water to the primer, according to the manufacturing company information then stored in the deep freeze. The primer that has been used was *spa*-1095F and *spa*-1517R [14, 15]. In order to use this diluted stock solution in PCR mixture, it must be diluted to get 10 pmol/ µl as a final concentration by adding 10 µl from the original stock solution to 90 µl of deionized distal water and stored in the deep freeze until its usage in the PCR mixture.Final volume 20 µl of PCR mixture was prepared (Master mix 12.5 µl, forward and reverse 1µl for each, nuclease-free water 7.5µl, and DNA 3µl), then short spin by microcentrifuge.

By Thermal Cycler System the DNA have been amplified according to the following program: Initial denaturation for 4 min at 95℃ for 1 cycle, Denaturation for 30 Sec. at 95℃ for 30 cycles, Annealing for 45 Sec. at 50℃ for 30 cycles, Extension for 45 Sec. at 72℃ for 30 cycles, Final extension for 7 min. at 72℃ for 1 cycle, and then Hold for 10 min. at 10℃ for 1 cycle. To confirm the presence of amplified DNA by PCR, agarose gel electrophoresis was adopted.

The PCR products were sent to Macrogen Corporation in Korea, and the results of sequencing *spa* gene were received through E-mail, and then analyzed by BioNumerics software in order to genotype *S. aureus* isolates and identify their *spa*-types.

Study the Antibiotic resistance for *S. aureus* isolates

An overnight bacterial broth culture (Nutrient broth) was prepared by using isolated colonies on mannitol salt agar. To start the Kirby-Bauer assay, first, a bacterial suspension was prepared by taking five to six bacterial colonies and suspend them in 5ml sterilized D.W then mix by a vortex in order to compare and adjust their turbidities to match 0.5 McFarland standard by adding more colonies or more D.W. 0.5McFarland standard is equivalent between 1 x 10^8 to 2 x 10^8 CFU/ml of a bacterial suspension. The assay was started by swabbing the prepared suspension on the Muller-Hinton agar plate three times all over its surface by rotating the plate 60° after each time, then the swab will pass round the edge of the agar surface. After the plates were dried at room temperature, antibiotic discs (table 1) were placed on the agar surfaces using sterile forceps. Incubation at 35°C for 24 h. and the results were recorded depending on standard interpretative measures of inhibition zone as shown in table 1[16, 17].

Table 1: Antibiotic disks used in disk diffusion test

Antibiotic	Disc content (µg unless stated)	Inhibition Zone Diameter interpretation(mm) R	I	S	Reference	Group
Gentamicin (CN)	10	≤12	13-14	≥15	(18)	Aminoglycoside
Amikacin(AK)	30	≤ 14	15-16	≥ 17	(18)	Aminoglycoside
Rifampin (RA)	5	≤ 16	17-19	≥20	(18)	Ansamycins
Ciprofloxacine(Cip)	5	≤ 15	16-20	≥21	(18)	Fluoroquinolones
Cephalothin (KF)	30	≤14	15-17	≥18	(19)	Cephalosporins 1st generation
Cefoxitin (Fox)	30	≤ 21	—	≥ 22	(18)	Cephalosporins 2nd generation
Cefotaxime (CTX)	30	≤14	15-22	≥23	(19)	Cephalosporins 3rd generation
Chloramphenicol(C)	30	≤ 12	13-17	≥18	(18)	Miscellaneous
Doxycycline (Do)	30	≤12	13-15	≥16	(18)	Tetracyclines
Vancomycin (VA)	30	--	—	≥15	(19)	Glycopeptides
Amoxicillin (AX)	25	≤21	22-27	≥28	(20)	Penicillins
Pencillin (P)	*10 U	≤28	—	≥29	(18)	Penicillins
Oxacillin (OX)	5	≤ 13	—	≥ 16	(20)	Penicillins
Trimethoprim(TMP)	10	19-26**			(21)	Folate pathway inhibitor

*U: Units; Penicillin is one of the few antibiotics that is still measured in terms of units rather than weight in milligrams or micrograms.

**Concentration of the Antibiotic provided by HiMedia as required by the users.

Results and Discussion

Antibiotic resistant pattern of different *spa*-type of *S. aureus*

Eighteen *spa*-types of *S. aureus* were detected, as illustrated in the table2 below

Table 2: Different *spa*-types isolated from Burn, wound, and hospital-environment samples

Number of isolates	The site of isolation	*Spa*-type
10	6 D.F	t037
	1 Medical waste at nursing cart	
	3 Burn injuries	
1	Burn injuries	t13157
1	Clean surface of nursing cart	t14870
1	Out patient with burn blister containing pus	t005
1	D.F	t223
1	After stabbing wound and surgery to the bladder	t386
1	bullet injury	t304
1	D.F	t304
1	D.F	t304

D.F: diabetic foot ulcer

All *S. aureus* isolates (18 isolates) showed 100% sensitivity to chloramphenicol, doxycycline and vancomycin.

The high sensitivity to chloramphenicol was also reported by Neha*et al*. when tested *S. aureus* from various skin samples [(22)]. In Afghanistan, a high percentage of *S. aureus* was found to be sensitive to doxycycline [(23)]. Furthermore, In 2013 Iraqi MS.C thesis, doxycycline sensitivity of *S. aureus* in wounds was different from that in burn isolates[(24)].

All the isolates were vancomycin sensitive in this study as well as in 2013 [(24)].

Sensitivity of trimethoprim, oxacillin and amoxicillin at higher concentrations than that determined by Clinical and laboratory standards institute (CLSI)

Four *S. aureus* isolates were sensitive to trimethoprim that used at concentration 10 µg, three of them (two *spa* type t304 and one t386) gave diameters 20, 28, and 29 mm of inhibition zone, while one isolate (t304) carried both *tst* and *sea* gave 32mm inhibition zone, this may be due to the increase of bacterial virulence that can cause a decrease in antibiotic resistance[(25)]. The remaining *S. aureus* isolates were more resistant (zero inhibition zone) to the 10 µg trimethoprim concentration

as compared with that corresponding inhibition zone diameter 19-26 mm that has been presented by Himedia[21]. A study on the resistance of *S. aureus* from skin samples gave a smaller inhibition zone (12mm) when trimethoprim was 10 µg[22].

Resistance to 5 µg of oxacillin was recorded in all *S. aureus* isolates which have a diameter outcome of zero mm except two *spa*-types t304 were (14 and 20 mm) and one t386 (10mm) as shown in figure 1. It was expected to see a 27-35mm diameter of inhibition zone instead of zero mm when testing *S. aureus* to 5 µg oxacillin, according to the inhibition zones presented by Himedia[21].

All the isolates were resistant to amoxicillin 25µg, by displaying no inhibition zone, although *S. aureus* inhibition zone was predicted as 28-36mm according to Himedia[21].

Table 3 shows the sensitivity of trimethoprim, oxacillin and amoxicillin at higher concentrations than that determined by CLSI.

Table 3: The resistance of *spa* type to TMP, AX, OX according to Fluka and Himedia

Number of isolates	*Spa*-type	TMP (10 µg) I.Z (mm)	OX (5 µg) I.Z (mm)	AX (25 µg) I.Z (mm)
10	t037	R **	R **	R **
1	t13157	R **	R **	R **
1	t14870	R **	R **	R **
1	t005	R **	R **	R **
1	t223	R **	R **	R **
1	t386	S (29 mm)	R(10mm)	R **
1	t304	S (28 mm)	R **	R **
1	t304	S (32 mm)	I (14mm)	R **
1	t304	S (20 mm)	S (20 mm)	R **

I.Z: diameter of inhibition zone, R **: resist with No inhibition zone; OX: oxacillin, Ax: amoxicillin, TMP: trimethoprim

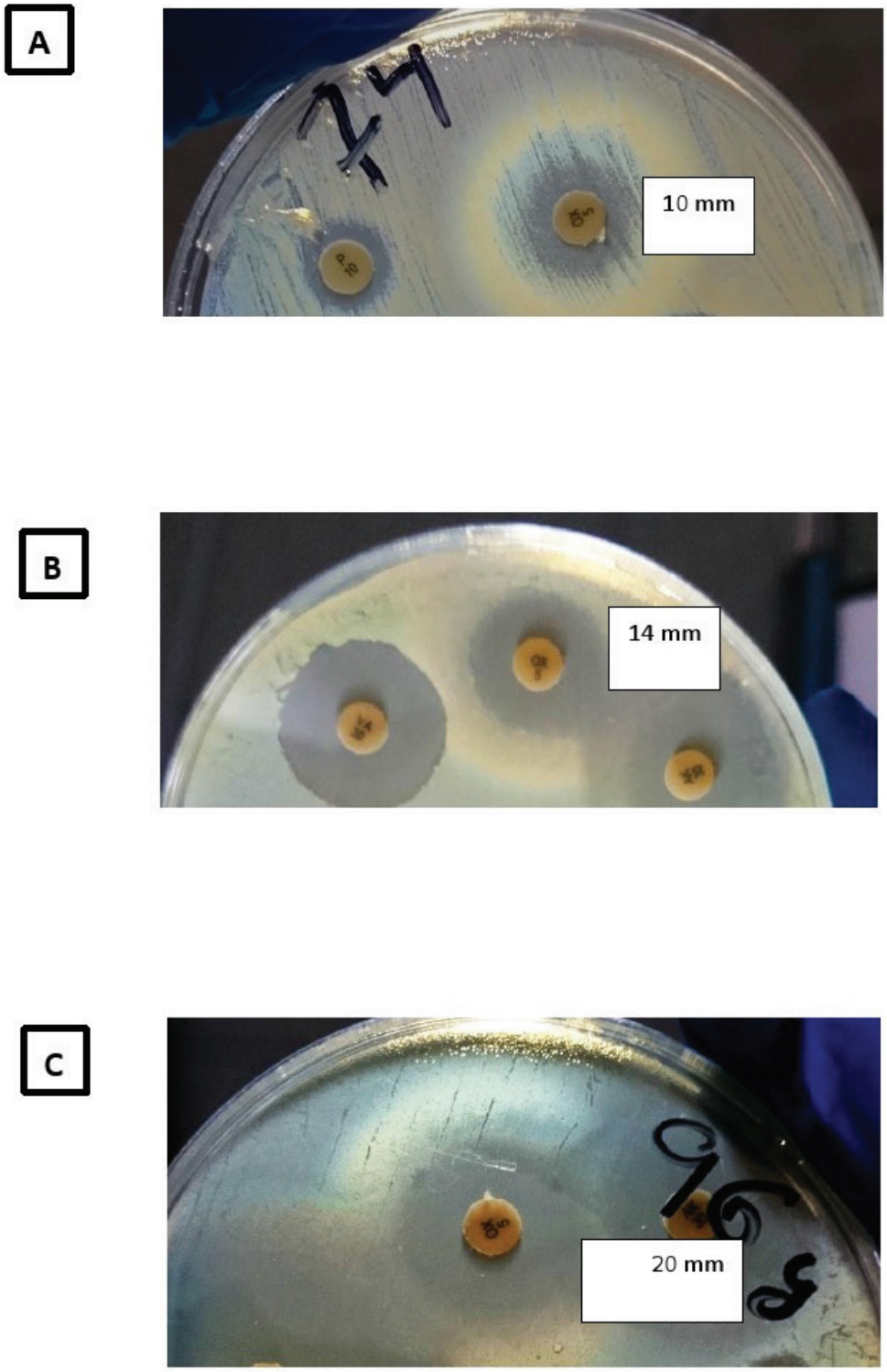

Figure 1 : Showing Inhibition zone to Oxacillin 5µg according to Fluka, A; t386 showing resistant to OX with 10mm inhibition zone, B; t304 showing intermediate resistant to OX with 14mm inhibition zone, C; t304 showing Sensitivity to OX with 20mm inhibition zone

Resistance pattern according to CLSI

Table 4 shows the resistance of the *S. aureus*to 8 antibiotics, it is clear that *spa*-type t037 is highly resistant to antibiotics and it shared the same resistance pattern with *spa*-type t13157 which differs from t037 by one repeat in X region of *spa* gene. While t005 that differs by its repeats has also shown a high resistance pattern which may be due to the source of the isolate that was from burn blister in outpatient, and it depends on the medications that the person was taking.

All three *spa*-type t304 showed lower resistance (resist 4 antibiotics) only one has additional resistance to rifampin with 16 mm inhibition zone which is the largest diameter to consider it resistant according to CLSI. The other types of *spa* (t223, t14870, and t386) were similar to t304 in their resistance pattern as it is shown in table 4.

According to the pattern of antibiotic resistance, the isolates can be divided into two groups; one is a multi-drug resistant group (t037, t13157, and t005) that resist 7 or 8 out of 11 antibiotics and categorized within five groups: penicillins, cephalosporins, fluoroquinolones, ansamycins, and aminoglycoside; while the other group was not considered as multi-drug resistant (t304, t386, t223, and t14870) due to its resistance to four or five antibiotics which were within penicillins and cephalosporins classes.

Table 4: Resistance pattern of different *spa*-types according to CLSI

Number of isolates	*Spa* type	Resistant pattern
1	t13157	CTX, P, Fox, KF, CN, Cip , AK, Ra
4	t037	CTX, P, Fox, KF, CN, Cip, AK, Ra
3	t037	CTX, P, Fox, KF, CN, Cip, (AK ^{I})*, Ra
3	t037	CTX, P, Fox, KF, CN, Cip, Ra
1	t005	CTX, P, Fox, KF, CN, Cip, AK
1	t386	CTX, P, Fox, KF, (AK ^{I})*
1	t304	CTX, P, Fox, KF, RA
2	t304	CTX, P, Fox, KF
1	t223	CTX, P, Fox, KF
1	t14870	CTX, P, Fox, KF

***^{I}: have Intermediate sensitivity; AK: Amikacin, Cip: ciprofloxacin, CN: gentamicin, RA: rifampin, KF: cephalothin, Fox: cefoxitine, P: penicillin, CTX: cefoxitine,**

Amikacin-resistant was noticed to be varied among the same *spa*-type, while all the isolates have the same resistance pattern for chloramphenicol, doxycycline, vancomycin, cephalothin, cefoxitin, penicillin, andcefotaxime. Therefore, the focus would be on three antibiotics: rifampin, gentamicin, and ciprofloxacin where the resistance varies according to the *spa*-types of *S. aureus*isolates.

Gentamicin and amikacin are aminoglycosides that were used in this study showed *S. aureus* had different resistance patterns: both gentamicin/amikacin resistant, both gentamicin/amikacin sensitive, gentamicin

sensitive/amikacin intermediate, gentamicin resist/ amikacin intermediate, and gentamicin resist/ amikacin sensitive. These variations in the resistant pattern could be due to differences in resistant-mechanisms depending on the plasmodial or chromosomic genetic elements. [26].

Many of *S. aureus*12/18 (66.66%) was noticed to be resistant to rifampin. Rifampin-resistant arises from a chromosome mutation. According to Guérillot*et al.* the emergence of many stable rifampin-resistant lineages among a global collection of *S. aureus* isolates might be due to the usage of this antibiotic [27].

Ciprofloxacin resistance is chromosomally mediated and not associated with plasmids. It rapidly developed in *S. aureus* after introducing the antibiotic [28], therefore many *S. aureus*(12/18) noticed to resist this antibiotic.

Peng*et al*. recorded t037 strain was sensitive to rifampin[29] and recently in Iran, rifampin sensitivity was recorded for t037 [30]. In California, a high sensitivity (98%) for both rifampin and gentamicin was observed [31]. On the contrary to the t037 isolated in this study were 100% resistant to rifampin.

Research conducted in Taiwan hospitals noticed that t037 from the skin was resistant 100% to ciprofloxacin and gentamycin [29], also it has been recorded a high percentage (80%) of resistance in Palestine [32], this was in agreement with the current study (100% resistant for both antibiotics).

An Italian thesis [33] studied the antibiotic resistance of t13157 that was isolated from clinical samples and found that it has a resistance to rifampin and gentamycin as that given in this study.

The single strain t005 of this study were resistant to gentamicin, amikacin, and ciprofloxacin. The same resistance was recorded in 66.7% of the isolated t005 in Iran [30].

Strain t304,and t386 showed sensitivity to ciprofloxacin, gentamycin and rifampin. This is similar to that reported in an Iranian study[34].

A strain t223 has been isolated and it showed ciprofloxacin, gentamycin, and rifampin sensitivity. According to research in Gaza [35], this strain that isolated from the nose of healthy peoples was sensitive to non-β-lactam antibiotics; including ciprofloxacin and gentamycin, also in Palestine,[32] t223 in different clinical samples had displayed low resistance to gentamicin (18.2%) and ciprofloxacin (9.1%), while in Iran[30] the resistance to rifampin was 26.7%.

t14870in this study was resistant only to the β-lactam group (cephalosporins and penicillins) as well as trimethoprim and was notconsidered as a multi-drug resistant strain. However, in another study [36] some (3/5) of t14870 isolates of animal origin were observed to be multi-drug resistant.

Conclusion

The most resistant group in this study have been isolated from burn patients (three t037,one t13157,one t005), diabetic foot ulcers (six t037) and medical waste at nursing cart (one t037) while the other group with less resistance were collected from diabetic ulcer at the clinic (two t304), bullet injury (one t304), clean surface nursing cart (t14870), after stabbing wound and surgery to the bladder (t386) and diabetic foot ulcer (t223) that is isolated from a person who was residing at a different sector than that where t037 has been detected. This indicates that people stayed for long period at hospitals to treat their injuries are more susceptible to be infected by more resistant strains, and therefore it is recommended to separate each patient that required a prolong treatment with commitments to hygiene controls to prevent the transmission of high-resistant strains among the patients.

Conflict of Interest: Nil

Source of Funding: Self

Ethical Clearance: Samples were collected from Al-Kindy and Al-Yarmouk teaching hospitals, Baghdad/ Iraq, after administrative approval.

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