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Vancomycin Resistance Among Methicillin-Resistant Staphylococcus Aureus Isolated from Clinical Samples in Erbil City-Iraq

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ABSTRACT

Bacterial isolates obtained from different sources of 348 human specimens including burn, wound, urine, and stool from the database of Internal Lab of Teaching Hospital, Irbil-Iraqi Kurdistan region, were collected from May 20, 2012, through January 19, 2013, of which 228 isolates were positive for Staphylococcus aureus.

Cultural studies were performed using different cultures and biochemical tests to ensure the identity of species under study. The susceptibility of the isolates for the antibiotics test were done using 22 different antibiotic disks including carbenicillin (CAR), vancomycin (VA), clindamycin (DA), methicillin (MY), cephalothin (KF), pipercillin (PRL), nitrofurantoin (F), cephalexin (CL), rifampicin (RA), gentamycin (G), chloramphenicol (C), trimethoprim – sulfamethoxazole (SXT), ceftazidime (CAZ), polymyxin B (PB), amoxicillin–cluvalinic acid (AMC), doxycycline (DO), amikacin (AK), oxacillin (OX), ciprofloxacin (CIP), cefixime (CFM), cefoperazone (CEP), and neomycin (NEO).

The results showed that resistance for the antibiotics ranged from 26.31% to 98.61% for DA and MY, respectively. A total of 78.94% of the isolates that demonstrated resistance to MY were also found to resist VA. Thus, we conclude that some strains of S. aureus isolates acquired genes that are able to resist those antibiotics.

Key words: Staphylococcus aureus, Biochemical Tests, Disk Diffusion of Antibiotic Susceptibility.

INTRODUCTION

The problem of the resistance of Staphylococcus aureus to antibiotics is rapidly growing (1). Antibiotic-resistant genes benefit bacteria enabling them to combat the deadly effect of the antibiotic. The question arises whether resistant bacteria suffer a cost of resistance in the absence of antibiotics. If so, the use of a particular antibiotic should be suspended until the genotype of the resistance is cleared or at least declined in frequency. Numerous studies indicate that resistant genotypes are less fit than the sensitive ones in the absence of antibiotics. However, these studies use nave bacteria that have no evolutionary background related to resistant bacteria. So, the question arises whether bacteria have the capability to adapt and overcome the side effects of the resistant genes. As a consequence, it will be extremely difficult to eliminate resistant genotypes simply by suspending the use of antibiotics (2). Resistance to antibiotics happens through several mechanisms as follows: production of enzymes, impermeability of bacterial outer membrane, alteration or overexpression of the drug target, enhanced efflux pump, alteration of metabolic pathway, and hiding antibiotic targets. The latter two mechanisms have been recently discovered (3, 4). A unique feature of the enzymes that alter the structure of antibiotics and render bacteria resistant to them is that these enzymes reduce the concentration of such drugs, and this property has been the biggest obstacle to anti-infection therapy for researchers and clinicians who are working on new approaches.

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A number of extracellular enzymes and exotoxins such as coagulase, alphatoxin, leukocidin, exfoliatins, enterotoxins, and toxic shock toxin are responsible for clinical symptoms of infections by this pathogen (5).

S. aureus are common colonizers of healthy humans; however, they can be opportunistic pathogens. They produce a range of potent protein-based enzymes (toxins) that may cleave host molecules or damage host cells (6).

VA-Resistant S. aureus (VRSA)

The emergence of high levels of penicillin resistance followed by the rapid evolution and spread of strains resistant to semisynthetic penicillin, macrolides, tetracycline, and aminoglycosides has made the treatment of staphylococcal disease a global challenge. In the 1980s,due to the widespread occurrence of methicillin-resistant S. aureus (MRSA), empiric therapy for staphylococcal infections was switched to VA in many health care institutions (7).

In this study we aimed to determine the prevalence of MRSA and VRSA/vancomycin-intermediate Staphylococcus aureus (VISA) by standard microbiological methods of susceptibility testing (disk diffusion) in clinical isolates of S. aureus in Erbil hospitals.

MATERIALS AND METHODS

Bacterial strains

This study is based on data gathered from 228 isolates of S. aureus that were identified by characteristic morphology, Gram stain, and biochemical tests.

Media, chemicals and reagents

The chemicals and reagents used were of analytical grade and were obtained from Sigma chemical co. (USA) and Oxoid Ltd. (UK). Media used in this study were: Nutrient, Blood, Mueller-Hinton and Mannitol Salt Agar. All media were prepared according to the manufacturers specifications and sterilized at 121C for 15 min at 15 lb/inch^2 pressure (8, 9).

Isolation and identification of isolates

Discrete colonies were subcultured onto fresh agar plates aseptically to obtain pure cultures of the isolates. All isolates were Gram stained to determine their gram category (10). Mannitol fermentation tests were carried out. Other tests including coagulase test, catalase test, urease test, oxidase activity, Voges-Proskauer (VP) test, motility agar test (9), Kliglers iron agar (KIA) test (11), and clumping factor A (ClFA) test (5) were also done.

Inoculum preparation

Five discrete isolates were inoculated into a nutrient broth of 5 mL and incubated at 35°C. A spectrophotometer was used to monitor the turbidity of the cultures. Immediately, the turbidity exceeded 0.5 McFarland of standard solutions (12), at which incubation was stopped. The broth culture then was diluted to give a count of approximately 1.5±108 CFU/mL.

Antibiotic susceptibility Test

Antibiotic susceptibility of S. aureus isolates was determined by the disk diffusion method using the following disks for all 228 isolates (Table 1): CAR 100 µg, VA 30 µg, DA 2 µg, MY 10 µg, KF 30 µg, PRL 100 µg, F 300 µg, CL 30 µg, RA 5 µg, G 10 µg, C 30 µg, SXT 1.25 + 23.75 µg, CAZ 30 µg, PB 300 µg, AMC 20 + 10 µg, DO 30 µg, AK 30 µg, OX 1 µg, CIP 5 µg, CFM 5 µg, CEP 75 µg, and NEO 30 µg. The cultures were overnight incubated and then recultured on Muller-Hinton agar. The standard antibiotic disks were used for direct inhibition tests. These studies were performed using standardized inoculums with selective media. Disks were directly applied on the cultured plates. After incubation for 24 hours, zones of bacterial inhibition were measured in millimeters for all tested disks.

RESULTS

Collection of S. aureus isolates Table 2 shows percentages of sources from which samples were collected. Wound represents 38.09%, urine 33.33%, burn 75.86%, and stool 75%.

Identification of S. aureus isolates

S. aureus grows on most bacteriologic media. Colonies of S. aureus on MSA (Mannitol Salt Agar) are of cream color and change the pink color of the medium to golden yellow, and these colonies are 34 mm, smooth, low convex, and opaque. Table 3 shows the results of biochemical tests that are done for the identification purpose. It is indicated that S. aureus is negative for oxidase test while it shows positive results for each of DNase, mannitol fermentation, blood hemolysis, urease, catalase, and coagulase tests.

TABLE 1: MAntibiotics, symbol, final concentration and diameter of inhibition zone (mm) against S. aureus (13, 14).

No.	Antibiotics	Symbol	Disk potency (µg or U)	Zone Diameter		
				Resistant	Intermediate	Sensitive
1	Amikacin	AK	30	≤14	15 – 16	≥17
2	Amoxicillin–clavulanic acid	AMC	20 + 10	≤13	14 – 19	≥20
3	Carbenicillin	CAR	100	≤13	14 – 16	≥17
4	Cefalexin	CL	30	≤14	15 – 17	≥18
5	Cefoperazone	CEP	75	≤15	16 – 20	≥21
6	Cefixime	CFM	5	≤15	16 – 18	≥19
7	Ceftazidime	CAZ	30	≤16	----	≥16
8	Cephalothin	KF	30	≤14	15 – 17	≥18
9	Chloramphenicol	C	30	≤12	13 – 17	≥18
10	Ciprofloxacin	CIP	5	≤21	22 – 24	≥25
11	Clindamycin	DA	2	≤14	15 – 20	≥21
12	Doxycycline	DO	30	≤12	13 – 15	≥16
13	Gentamycin	G	10	≤15	----	≥15
14	Methicillin	MY	10	≤9	10 – 13	≥14
15	Neomycin	NEO	30	≤12	13 – 16	≥17
16	Nitrofurantoin	F	300	≤14	15 – 16	≥17
17	Oxacillin	OX	1	≤12	13 – 15	≥16
18	Pipercillin	PRL	100	≤17	18 – 20	≥21
19	Polymyxin B	PB	300	≤8	9 – 11	≥12
20	Rifampicin	RA	5	≤16	17 – 19	≥20
21	Trimethoprim–Sulphamethaxazole	SXT	1.25 + 23.75	≤10	11 – 15	≥16
22	Vancomycin	VA	30	≤14	15 – 16	≥17

Antibiotic susceptibility of S. aureus isolates

Table 4 illustrates the susceptibility test of all 228 isolates of S. aureus done against 22 widely used antibiotics. The results showed a wide spectrum of resistance to antibiotics. The highest resistance percentage was 100.0% for each of G, CFM, and CEP, and the lowest resistant percentage was 26.31% to DA. Patients under study were admitted in the hospital and were not subjected to any antibiotic treatment.

DISCUSSION

S. aureus is an opportunistic pathogen that causes human infections. Morphological, cultural, and biochemical features were investigated using API Staph strip. Okonko (15) demonstrated that S. aureus is positive for catalase, coagulase, urea utilization, mannitol fermentation, and hemolysis, while culturing on Kliglers iron agar, isolates change the medium color yellow in both slope and butt and have the ability to

TABLE 2: Distribution of S. aureus isolates according to their sources.

Specimens	No. of samples	No. of positive samples	Percent of positive samples
Wound	126	48	38.09
Urine	36	12	33.33
Burn	174	132	75.86
Stool	48	36	75
Total	384	228	59.37

TABLE 3: Biochemical tests result of S. aureus isolates.

No	Biochemical tests		Results
1	Gram stain		+
2	DNase		+
3	Mannitol fermentation		+
4	Blood hemolysis		α-Hemolysis
5	Urease		+
6	Catalase		+
7	Coagulase		+
8	Oxidase		-
9	Kligler's iron test	Slope	Yellow
		Butt	Yellow
		Hydrogen sulphide (H_2S)	+
		Gas production	-/G

+: positive results; -: negative results.

produce H2S and gas. To support this, the API Staph strip was performed for 20 isolates of S. aureus, and it showed negative results for oxidase. All isolates of S. aureus showed different percentiles against all 22 antibiotics starting from 26.31% against DA and the highest level of resistance 100% against G,OX,CFM,and CEP. Tagoe (16) points that all isolates (8 isolates among 14 different bacterial genera) of S. aureus showed resistance percentage of 62.5% to each of AP, P, FIX, ERY, CRX, and COT, and 50% to CTX and CX. Prabhu (17) tested the antibiotic susceptibility for 20 isolates of S. aureus and concluded that there was an inducible DA resistance that is supported by Vivek (18) who reports that 41 out of 87 clinical isolates of S. aureus showed inducible DA resistance. Okonko (15) detected that S. aureus resisted to AM and VA with 81.8% and 40.6%, respectively. zelik (19) confirmed that 65 isolates of S. aureus showed 100% resistance for VA antibiotic. However, Anywar (20) tested susceptibility for 1370 isolates of S. aureus,and among these isolates 70.95% resist to AMP, 32.7% to C, 1.3% to CIP, 7.05% to E, 1.3% to ME, 42.55% to TE, and 49.15% to CT while all isolates were susceptible to G. Duran (25) tested susceptibility of 139 isolates of S. aureus against ten antibiotics and found that the highest resistance percentage was 60.4% for Erythromycin,the lowest percentage was 16.5% for Methicillin, and all isolates were sensitive to Vancomycin. Saderi (7) illustrated that the resistance percentages of 238 isolates of S. aureus against 9 antibiotics were 91.1,58.9,56.7,42.3,33.1, 30.0, 29.8, 15.5, and 18.4% for P, OX, AMC, TE, E, G, CEP, DA, and IMP respectively. Nkwelang (22) clarified that the results of susceptibility test of 85 isolates of S. aureus against 12 antibiotics were 100% for P and AMP,94.1% for ME,83.5% for G, 75.3% for OX, 69% for CRO, 38.8% for DO, 22.4% for SXT and E, 20.0% for CIP, 12.9% for OX, and 8.2% for VA. Edelmann (23) found that the resistance percentage of 71 isolates of S. aureus were 91.1% for PRL and 98.2% for KF. Daza (24) were performed antibiotic sensitivity for 749 of bacterial isolates of S. aureus and among of them 43 isolates (represent 5.74%) record 100% resistance to F (Nitrofurantoin) antibiotic. Over 90% of S. aureus were resistant to penicillin.

TABLE 4: Resistance of S. aureus isolates to antibiotics.

No	Antibiotics	S. aureus	
		No. of resistant isolates	Resistance percentage
1	AK	38	50
2	AMC	44	57.89
3	CAR	72	94.73
4	CL	41	53.94
5	CEP	76	100
6	CFM	76	100
7	CAZ	56	73.68
8	KF	40	52.63
9	C	72	94.73
10	CIP	60	78.94
11	DA	20	26.31
12	DO	72	94.73
13	G	76	100
14	MY	71	98.61
15	NEO	51	67.1
16	F	24	31.57
17	OX	76	100
18	PRL	72	94.73
19	PB	24	31.57
20	RA	64	82.21
21	SXT	72	94.73
22	VA	60	78.94

*: Abbreviation is given in Table 1.

Emergence of VA-Resistant S. aureus VRSA

VA has been the most reliable therapeutic agent against infections caused by MY-resistant S. aureus (MRSA). Table 4 shows that 98.61% of all isolates were resistant to ME (Methicillin antibiotic) antibiotic and 78.94% of the isolates were resistant to VA antibiotic.

The mechanism of VA resistance in S. aureus is not well understood yet. It was initially thought that all the VRSA

isolates would acquire the vanA and mecA genes that code for VA resistance in Enterococcus species. Further, VA-resistant Enterococcus faecalis emits a sex pheromone that promotes plasmid transfer, and it has recently been demonstrated that this same pheromone is produced by S. aureus. The emission of this pheromone by S. aureus organisms that are in proximity to VA-resistant enterococci that contain plasmids encoding vanA genes could result in the transfer of these resistance genes. However, thus far, neither the vanA genes nor their altered peptidoglycan products have been recovered in VAintermediate or VA-resistant S. aureus isolates. Instead, it appears that VA resistance in S. aureus is conferred by other alterations in the bacterial cell wall (25, 26).

Daum engineered laboratory strains of VISA and VRSA that had much thicker cell walls than the sensitive parent strains. Subsequent investigators demonstrated that cell wall synthesis and turnover are unregulated in VRSA isolates, leading to thicker and more disorganized cell walls. Further, it appears that resistant isolates have significantly less cross-linking in the peptidoglycan component of the cell wall. To exert an effect, VA must reach the cytoplasmic membrane and bind with nascent cell wall precursors, thereby inhibiting their incorporation into the growing cell wall. It has been proposed that the thicker, disorganized cell walls can actually trap VA at the periphery of the cell, thereby blocking its action. In fact, it has been shown that VA can be recovered intact from the cell walls of VISA and VRSA isolates, indicating that the antibiotic is not being inactivated but merely sequestered by the bacteria. Furthermore, the altered cell walls appear to have a reduced affinity for VA, as soluble targets are able to bind more antibiotic in the presence of vVA-resistant isolates (27).

Two enzymes located in the cytoplasmic membrane glycosyl transferase and transpeptidase assemble the murein monomer into a gigantic structure of peptidoglycan. Glycosyltransferase polymerizes murein monomers between their amino sugar moieties to produce nascent peptidoglycan chains. Then, transpeptidase, also known as penicillin-binding protein (PBP), links the newly formed nascent peptidoglycan chains to pre-existing peptidoglycan layers of S. aureus cells. In this step, PBP recognizes D-alanyl-D-alanine residues of murein monomer, cuts in between the two D-alanines, and ligates penultimate D-alanine to the tip of a pentaglycine chain protruding from pre-existing peptidoglycan layers. When the interpeptide bridge is formed, the terminal D-alanine of the murein monomer is lost from the completed peptidoglycan. However, it is known that about 20% of D-alanyl-D-alanine residues remain unprocessed by PBPs. As a result, many D-alanyl-D-alanine residues remain in the cell wall of a single S. aureus cell. PBP is the target of beta-lactam antibiotics such as penicillin. Beta-lactam is a structural analogue of D-alanyl-D-alanine, and it covalently binds to the S. aureus PBP at its D-alanyl-D-alanine binding pocket. This inactivates the PBP and inhibits the cross-bridge formation step of peptidoglycan synthesis, causing the cell to rupture from the peptidoglycan mesh. However, MRSA produces a unique PBP, designated PBP2 (or PBP2A), which has an extremely low binding affinity to beta-lactam antibiotics. As a result, the PBP2 can keep on synthesizing the peptidoglycan even in the presence of beta-lactam antibiotics. This is the basis of the beta-lactam resistance of MRSA. The unique PBP2 is the product of the exogenous gene called mecA carried by a mobile genetic element, SCCmec, which S. aureus has acquired from a yet unknown bacterial species by lateral gene transfer (28). The most variable feature of the VRSA genome is its plasmid content. In all cases, Tn1546 resides on a plasmid, even though it clearly transposed upon entry into some strains, and because of size, the chromosome would seem to be the most probable target for transposon insertion. The basis for the insertion site preference for plasmids over the S. aureus chromosome and also for an apparent incompatibility between the enterococcal Inc18 plasmid (played a major role in the Michigan outbreak) and an endogenous S. aureus pSK41 plasmid (present in several recipients) is unknown. VRSA genomes are replete with plasmids of enterococcal origin, highlighting their co-occurrence in polymicrobic infections and possibly in other ecologies. The multiplicity of plasmid structures conveying Tn1546, including S. aureus/enterococcal cointegrate plasmids, increases the odds of future transfers, possibly into staphylococcal lineages or species where a lower fitness cost is incurred (29).

However, in 1996 the first MRSA to acquire resistance to VA was isolated from a Japanese patient. In 2002, the first clinical isolate of VA-resistant S. aureus was reported in the United States (30). Our results demonstrated that the 78.94% of isolates (which resist 98.61% against Methicillin show resistance against VA (Table 4). Over the last decade, MRSA

strains had become endemic in hospitals worldwide. Our results are supported by both Edelmann (24) who reported that among 71 isolates of S. aureus, 99.2% were resistant to VA, and Daza (31) who demonstrated the same results, indicating that 100% of all isolates resist to VA antibiotic.

CONCLUSION

In conclusion, our results show the increase of VA resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity, which call for further epidemiological studies.

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