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مقاومة الفانكومايسين في المكورات العنقودية الذهبية المقاومة للمثيسلين و المعزولة من العينات السريرية في مدينة أربيل، العراق

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الملخص

الهدف: تحديد انتشار مقاومة الفانكومايسين في المكورات العنقودية الذهبية المقاومة للمثيسيلين VRSA بين المرضى الوافدين الى المستشفى التعليمي في اربيل ومقلومة هذه العزلات لنواع مختلفة من المضادات الحياتية.

المواد و الطرائق العمل: تم التاكد من تشخيص العزلات باستخدام اوساط زرعية مختلفة واجراء الاختبارات البايوكيميائية. كما تم اجراء اختبار الحساسية والمقاومة للمضادات الحياتية المختلفة باستخدام طريقة انتشار الاقراص.استخدمت اثنان و عشرون مضاد حيوي والتي شملت (كربنسيلين (CAR)، فانكوميسين (VA)، كليندامايسين (DA) ، مثيسلين (MY)، سيفالوثين (KF)، بيبرسيلين (PRL)، نايتروفورانتين (F)، سيفالكسين (CL)، ريفامبيسين (RA) ، جنتامايسين (G)، كلورامفينيكول (C)، ميثوبريم– سلفاميثوكسازول (SXT)، سيفتازيديم (CAZ)، بوليميكسين (PB)، أموكسيسيلين– حامض كلوفالينيك (AMC) ، دوكسي سايكلين (DO)، أميكاسين (AK)، أوكساسيلين (OX)، سيبروفلوكساسين (CIP)، سيفيكسيم (CFM)، سيفوبيرازون (CEP) ونيومايسين (NEO).

النتائج: اظهرت النتائج بان مقاومة عزلات VRSA للمضادات الحيوية تراوحت بين ٢٦,٣١٪ إلى ٩٨,٦١٪ ل DA وMY على التوالي. كما وجد بان ٧٨,٩٤٪ من العزلات المقاومة لـ MY كانت ايضا مقاومة لـ VA . تبين من نتائج هذه الدراسة بان بعض سلالات من بكتريا المكورة العنقودية المعزولة قد تمتلك جينات القادرة على مقاومة تلك المضادات الحيوية.

الاستنتاجات: اظهرت نتائج الدراسة الحالية زيادة مقاومة فانكوميسين بين المكورات العنقودية المقاومة للمثيسيلين MRSA نتيجة للستخدام المفرط للمضادات الحيوية الامر الذي يدعو إلى إجراء المزيد من الدراسات الوبائية .

كلمات المفتاحية: المكورات العنقودية الذهبية، اختبارات الكيمياء الحيوية، المضادات الحيوية.

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Vancomycin Resistance among Methicillin Resistant *Staphylococcus aureus* isolated from Clinical Samples in Erbil City, Iraq

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ABSTRACT

Objective: *To determine the prevalence of VRSA among patients attending Erbil Teaching Hospital and their resistance to community used antibiotics.*

Materials and methods: *Cultural studies using different cultures and biochemical tests were done to ensure the identity of species under study. Susceptibility of the isolates for the antibiotics test were done using discs of twenty two different antibiotic discs including (Carbenicillin (CAR), Vancomycin (VA), Clindamycin (DA), Methicillin (MY), Cephalothin (KF), Pipercillin (PRL), Nitrofurantoin (F), Cephalexin (CL), Rifampicin (RA), Gentamycin (G), Chloramphenicol (C), Trimethoprim–Sulphamethaxazole (SXT), Ceftazidime (CAZ), Polymyxin B (PB), Amoxicillin–Clavulanic acid (AMC), Doxycycline (DO), Amikacin (AK), Oxacillin (OX), Ciprofloxacin (CIP), Cefixime (CFM), Cefoperazone (CEP) and Neomycin (NEO).*

Results: *The results show that resistance for the antibiotics ranged from 26.31% to 98.61% for DA and MY consecutively. 78.94% of those demonstrated resistance to MY have also found resist to VA antibiotic. Thus, the current study concludes that some strains of S. aureus isolates acquired genes that are able to resist those antibiotics.*

Conclusions: *In conclusion, the results of the current study showed an increase of Vancomycin resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity, which call for further epidemiological studies.*

Keywords: *Staphylococcus aureus, Biochemical Tests, Antibiotics.*

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1. Introduction

Staphylococcus aureus **are common colonizers of healthy humans; however, they can be opportunistic pathogens. It produces a range of potent protein-based enzymes (toxins) that may cleave host molecules or damage host cells [1, 2].**

The problem of the resistance of *S. aureus* to antibiotics is rapidly growing [3]. Antibiotic resistant genes benefit bacteria enabling to combat the deadly effect of the antibiotic. The question that arises is that, do bacteria suffer in the absence of antibiotics? If so, suspension of the usage of a particular antibiotic until the phenotype of the resistance is cleared or at least declined in frequency. Numerous studies indicate that resistant phenotypes are less fit than the sensitive once in the absence of antibiotics. As a consequence, it will be extremely difficult to eliminate resistant genotypes simply by suspending the use of antibiotics [4]. Resistance to antibiotics happens throughout several ways, these mechanisms are: production of enzymes, bacterial outer membrane impermeability, alteration or over expression of the drug target, enhanced efflux pump, alteration of metabolic pathway, and hiding the antibiotic targets. The latter two mechanisms are of recently discovered [5, 6]. 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 have been the biggest obstacle encountered by the researchers and clinicians considering new approaches.

1.1 Vancomycin Resistant *S. aureus* (VRSA)

The emergence of high levels of penicillin resistance followed by the rapid evolution and spread of strains resistant to the semisynthetic penicillin, macrolides, tetracycline, and aminoglycosides has made 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 changed to Vancomycin in much health care institutions [7].

In this study we aimed to determine prevalence of MRSA and VRSA by standard microbiological methods of susceptibility testing (disk diffusion) in clinical isolates of *S. aureus* in Erbil hospitals.

2. Materials And Methods

2.1 Bacterial strains

This study is based on data gathered from 228 of S. aureus that identified by morphological characteristics, gram stain, and biochemical tested in internal lab of Erbil Teaching Hospital.

2.2 Media, chemicals and reagents

The chemicals and reagents used were of analytical grade, obtained from Oxoid Ltd. (UK). Media used in this study are: Nutrient, Blood, Mueller Hinton and Mannitol Salt Agar. All media were prepared according to the manufacturer's specification and sterilized at 121 °C for 15 min at 15 Ib/inch2 pressures [8, 9].

2.3 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, Catalase, Urease activity, Oxidase, Vogues–Proskauer (VP), Motility agar test [9], Kligler's Iron Agar (KIA) [11] and Clumping factor A (ClFA) test [5] were done as well.

2.4 Inoculum preparation

Five discrete isolates were inoculated into 5 ml of the Nutrient broth 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.

2.5 Antibiotic susceptibility Test

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

3. Results

3.1 Collection of *S. aureus* isolates

S. aureus isolates were mostly isolated from patients with wound and burn infections (41%), but their prevalence were fewer in patients with other cases (6%-17%). Table 2 elucidates the prevalence of S. aureus according to site of infection for each case. Wound represents (38.09%), urine (33.33%), burn (75.86%), and stool (75%).

Kirkuk University Journal /Scientific Studies (KUJSS)
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Table 1: Antibiotics, Symbol, Final concentration and Diameter of Inhibition zone (mm) against *S. aureus* [13, 14]

Antibiotics	Symbol	Disk potency (µg or U)	Zone Diameter		
			R*	I	S
Amikacin	AK	30	≤ 14	15 – 16	≥ 17
Amoxicillin - Clavulanic acid	AMC	20+10	≤ 13	14 – 19	≥ 20
Carbenicillin	CAR	100	≤ 13	14 – 16	≥ 17
Cephalexin	CL	30	≤ 14	15 – 17	≥ 18
Cefoperazone	CEP	75	≤ 15	16 – 20	≥ 21
Cefixime	CFM	5	≤ 15	16 – 18	≥ 19
Ceftazidime	CAZ	30	≤ 16	----	≥ 16
Cephalothin	KF	30	≤ 14	15 – 17	≥ 18
Chloramphenicol	C	30	≤ 12	13 – 17	≥ 18
Ciprofloxacin	CIP	5	≤ 21	22 – 24	≥ 25
Clindamycin	DA	2	≤ 14	15 – 20	≥ 21
Doxycycline	DO	30	≤ 12	13 – 15	≥ 16
Gentamycin	G	10	≤ 15	----	≥ 15
Methicillin	MY	10	≤ 9	10 – 13	≥ 14
Neomycin	NEO	30	≤ 12	13 – 16	≥ 17
Nitrofurantoin	F	300	≤ 14	15 – 16	≥ 17
Oxacillin	OX	1	≤ 12	13 – 15	≥ 16
Pipercillin	PRL	100	≤ 17	18 – 20	≥ 21
Polymyxin B	PB	300	≤ 8	9 – 11	≥ 12
Rifampicin	RA	5	≤ 16	17 – 19	≥ 20
Trimethoprim – Sulphamethoxazole	SXT	1.25+23.75	≤ 10	11 – 15	≥ 16
Vancomycin	VA	30	≤ 14	15 – 16	≥ 17

*: R = Resistant, I = Intermediate, S = Sensitive

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

Specimens	No. of samples	No. of positive samples	% 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**

Kirkuk University Journal /Scientific Studies (KUJSS)
Volume 12, Issue 2, March 2017
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3.2 Identification of *S. aureus* isolates

***S. aureus* grows on most bacteriological media. Colonies of *S. aureus* on MSA (Mannitol Salt Agar) are cream colored and change the pink color of medium to golden yellow, 3–4 mm, smooth, low convex, and opaque. Table 3 shows the results of biochemical tests that are done for identification purpose. It is indicated that *S. aureus* is negative for oxidase test while it shows positive result for each of DNase, Mannitol fermentation, blood hemolysis, urease, catalase, and coagulase tests.**

Table 3: Biochemical Tests Result of *S. aureus* isolates

*: + = Positive results; – = Negative results.

Biochemical tests		Results
Gram stain		+*
DNase		+
Mannitol fermentation		+
Blood hemolysis		α and β– Hemolysis
Urease		+
Catalase		+
Coagulase		+
Oxidase		–
Kligler's Iron test	Slope Butt Hydrogen Sulphide H_2S Gas production	Yellow Yellow + – /G

Kirkuk University Journal /Scientific Studies (KUJSS)
Volume 12, Issue 2, March 2017
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3.3 Antibiotic resistance of *S. aureus* isolates

Table 4 illustrates the susceptibility test of all 228 isolates of *S. aureus* against antibiotics.

Table 4: Resistance of *S. aureus* isolates to antibiotics

Antibiotics	*S. aureus*	
	No. of resistant isolates	% of resistant
AK*	114	50.00
AMC	132	57.89
CAR	216	94.74
CL	123	53.95
CEP	228	100.00
CFM	228	100.00
CAZ	168	73.68
KF	120	52.63
C	216	94.74
CIP	180	78.95
DA	60	26.32
DO	216	94.74
G	228	100.00
MY	213	93.42
NEO	153	67.11
F	72	31.58
OX	228	100.00
PRL	216	94.74
PB	72	31.58
RA	192	84.21
SXT	216	94.74
VA	180	78.95

*: Abbreviation is given in Table (1).

The results show a wide spectrum of resistance to antibiotics. Highest resistance 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.

4. Discussion

S. aureus **is notorious for its ability to become resistant to antibiotics. Infections that are caused by antibiotic resistant strains often occur in epidemic waves that are initiated by one or a few successful clones [15].**

All isolates of *S. aureus* show different percentage against all twenty two 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* have shown resistance percentage of 62.5% to each of AP, P, FIX, ERY, CRX and COT, 50% to CTX and CX. Prabhu [17] tested the antibiotic susceptibility for twenty isolates of *S. aureus* and concluded that there was an inducible clindamycin resistance which is supported by Vivek [18] who reports that among forty one out of eighty seven clinical isolates of *S. aureus*, show inducible –clindamycin resistance. Okonko [19] detected that *S. aureus* resist to AM and VA with 81.8% and 40.6% respectively. Özçelik [20] confirmed that 65 isolates of *S. aureus* show 100% resistance for VA antibiotic. However, Anywar [21] 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 susceptible to G. Duran [22] 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. Nkwelang [23] clarifies that the results of susceptibility test for 12 antibiotics for 85 isolates of *S. aureus* were 100% to P and AMP, 94.1% to ME, 83.5% to G, 75.3% to OX, 69% to CRO, 38.8% to DO, 22.4% to SXT and E, 20.0% to CIP, 12.9% OX and 8.2% to VA. Edelmann [24] has found that the resistance percentage of 71 isolates of *S. aureus* were 91.1% to PRL, and 98.2% to KF. Daza [25] 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.

4.1 Emergence of Vancomycin Resistant *Staphylococcus aureus* VRSA

VA antibiotic has been the most reliable therapeutic agent against infections caused by methicillin resistant *S. aureus* (MRSA). Table 4 shows that 98.61% of all isolates were resist to Methicillin antibiotic, 78.94% of these isolates resist to VA antibiotic.

The mechanism of Vancomycin resistance in *S. aureus* is not well understood yet. It was initially thought that all the VRSA isolates would acquire the *vanA* and *mec*A genes that codes for Vancomycin resistance in *Enterococcus* species. Further, Vancomycin 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*. Emission of this pheromone by *S. aureus* organisms that are in proximity to Vancomycin resistant enterococci that contain plasmids encoding *vanA* genes could result in transfer of these resistance genes. However, thus far, neither the *vanA* genes nor their altered peptidoglycan products have been recovered in Vancomycin intermediate or resistant *S. aureus* isolates. Instead, it appears that Vancomycin resistance in *S. aureus* is conferred by other alterations in the bacterial cell wall [26, 27].

Daum have engineered laboratory strains of VISA and VRSA that had much thicker cell walls than the sensitive parent strains. Subsequent investigators have 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. In order to exert an effect, vancomycin 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 vancomycin at the periphery of the cell, thereby blocking its action. In fact, it has been shown that vancomycin 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 vancomycin as soluble targets are able to bind more antibiotic in the presence of Vancomycin resistant isolates [28].

MRSA produces a unique penicillin binding protein (PBP), designated PBP2*α*, which has an extremely low binding affinity to beta lactam antibiotics. As a result, the PBP2*α* can keep on synthesizing the peptidoglycan even in the presence of beta-lactam antibiotics. This is the basis of beta lactam resistance of MRSA. The unique PBP2*α* is the product of the exogenous gene called *mecA* carried by a mobile genetic element, SCC*mec*, which *S. aureus* has acquired from an as yet unknown bacterial species by lateral gene transfer [29].

The most variable feature of the VRSA genome 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 that 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 Tn*1546*, 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 [30].

The results of the current study showed that the 78.94% of isolates (which resist 98.61% against Methicillin antibiotic) show resistant against Vancomycin (VA) (Table 4). Over the last decade, methicillin resistant *S. aureus* (MRSA) strains had become endemic in hospitals worldwide. Our results are supported by each Edelmann (24) which was reported that among 71 isolates of *S. aureus*, 99.2% were shown resistance against VA, and Daza [25] illustrating that same results were obtained, indicating that 100% of all isolates resist to VA antibiotic.

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