PREVALENCE OF VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* (VRSA) IN SOME EGYPTIAN HOSPITALS

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ABSTRACT

A long time ago, we found that increase in the prevalence of antibiotic resistant pathogens and strains in serious infections, the reason of distribution of these strains is because of the miss use of antibiotics to treat humans against different microorganisms,one of the most important infectious etiological agent is *Staphylococcus aureus*.*Staphylococcus aureus* strains were recovered from approximately 514 clinical samples were collected from patients admitted to Beni-Suef Public Hospitals,Assuit University and Beni-Suef University Hospital,Demerdash Hospital,S. Galal Hospital.Vancomycin resistance was determined by broth dilution method. Resistance against different antibiotics were determined by disc diffusion method. Screening for virulence genes were performed by PCR method.we found that From 514 clinical samples we found that *staphylococcus* strain were 308 strains (59.9%),and we found that *staphylococcus aureus* strain were 296 strains (96.1 %). Methicillin resistant *staphylococcus aureus* (MRSA) strain were 215 (72.6 %). Collected MRSA strains were distributed as 184 VSSA (85.5%),19VISA (8.8 %), and12 VRSA strains (5.5 %).The incidences of VRSA in hospitalized sample equal to non-hospitalized sample.The resistant genes detected from 31 strain (VISA and VRSA) were Mec A in 15 isolates (48.3 %),Van A in 12 isolates (38.7%), Panton valentine lucocidine toxin (lucotoxin) in 16 isolates (51.6 %), Enterotoxin type A in 18 isolates (58 %) followed by TSST in 14 isolates (45.1 %), and the lower incidences observed in genes of Exfoliative toxin type A in 5 isolates (16.1 %) and Exfoliative toxin type B in 1 isolate (3.22 %).The results of study provide that the high prevalence of VRSA in Egypt, andthe necessity for new and effective drugs against VRSA.

Keywords: *Staphylococcus aureus*,Methicillin-resistant, Antibiotic resistance Methicillin resistant *Staphylococcus aureus* (MRSA),Minimum inhibitory concentration (MIC), Vancomycin intermediate *S. aureus* (VISA), Vancomycin resistant *S. aureus* (VRSA), Vancomycin sensitive *S. aureus* (VSSA).

1-INTRODUCTION

A long time ago, we found increase in the prevalence of antibiotic resistant pathogens and strains in serious infections **(Brown *et al*.,2005;Jones *et al*.,2008;Chessa *et al*.,2015).**

The reason of distribution of these strains is due to the extensive and miss use of antibiotics in human and animal infections.Oneof the most important infectious etiological agents is *Staphylococcus aureus* **(Hany *et al*.,2020).***Staphylococcus aureus* especially methicillin-resistant *S. aureus* (MRSA), isemerging as a major public health problem in hospital and community settings, causing a lot of diseases. The emergence and spread of both health care and community-associated MRSA has made infection control intervention and treatment challenging **(Lowy *et al*.,1998).**There are different methods for detection of MRSA including minimum inhibitory concentrations (MIC), disc diffusion testing and the oxacillin screening agar method, The major problem in routine screening is that MRSA strains are heterogeneous in the expression of resistance to β-lactam agents **(Velasco *et al*.,2005).**Vancomycin is one of the oldest antibiotics, and has been in clinical use for nearly 60 years. Dr. Kornield, an organic chemist of Eli Lilly, isolated vancomycin from *Streptomyces orientalis* in the deep jungles of Borneo in 1957. Vancomycin is highly active against Gram-positive bacteria, such as *Staphylococci, Enterococci, Corynebacterium, Streptococci, Pneumococci, Listeria, and Clostridia* (**Rubinstein *et al*.,2014).**Vancomycin has been considering the most reliable therapeutic agent against infections caused by MRSA.There has been an alarming emergence of vancomycin-resistant *S. aureus* (VRSA), possibly due to: (1) the widespread use of vancomycin to treat infections caused by MRSA; (2) a patient's immune status; (3) surgical procedures; and (4) involvement of healthcare workers infected with MRSA **(Kaiser *et al.*,2011;Schweizer *et al*.,2012;Chen *et al*.,2012).**

In1996,VISA (vancomycin-intermediate *S. aureus*) was first discovered in Japan **(Hiramatsu *et al*.,1997).** In 2002 vancomycin-resistant *S. aureus* (VRSA) was first reported in the USA **(Howden *et al*.,2004).**Subsequent isolation of VRSA from different countries has confirmed that the emergence of these strains is a global issue **(Chang *et al*.,2003; Tenover *et al*.,2004; Saha *et al*.,2008).**Due to the prevalence of the VRSA infection cases, there is notreatment guideline is available. Out of all reported VRSA cases, only a few provided detailed clinical data. VRSA isolates commonly remain susceptible to multiple antimicrobial agents. Itwas reported that VRSA isolates were susceptible to ceftaroline, daptomycin, linezolid, minocyline, tigecycline, rifampin, and trimethoprim/sulfamethoxazole. Therefore, a systemic antimicrobial therapy with effective antibiotics is generally implemented upon VRSA isolation determination by a clinical laboratory. Wound care bacterial colonization is the first step in development of infections. Wound is the most common source of VRSA isolates and provides an environment for co-infection and co-colonization of MRSA and VRSA **(Yanguang Cong *et al.*,2020).**There are numerous virulence factors produced by *S.aureus*, including Panton-Valentine Leukocidin (PVL), which is a pore forming cytotoxin more often identified in community-associated MRSA strains than hospital-associated strains **(Lina.,1999).**Therefore, this study was conducted to examine the prevalence of VRSA in some of the Egyptian Hospitals.

2- Materials and methods

2.1. Study area and sampling

Different clinical Samples (pus, sputum, ear discharge, nasal discharge and urine) were collected from patients admitted to Beni-Suef Public hospitals,Assuit University and Beni-Suef University hospital in sterile containers according to standard laboratory procedures and transferred in a transport media(AMIES) for processing and isolation in microbiology lab, Faculty of Pharmacy, Beni-Suef University. Each sample was transferred to sterile brain-heart infusion broth and incubated for 24 hr at 37^{o}C. After 24 hr the samples were sub cultured on mannitol salt agar at 37^{o}C for 24-48hr. The colonies suspected of being *Staphylococcus aureus* were identified by standard procedures **(Cappuccino *et al*.,1996;Wibbenmeyer *et al*.,2010).**

2.2. Isolation and identification of *Staphylococcusaureus*

A pure colony was selected and subjected to Gram staining, for microscopic observation.Required confirmatory biochemical tests including catalase, tube coagulase, slide coagulase, blood hemolysis and DNase tests were performed to identify suspected *S. aureus* **(Cappuccino *et al*.,1996).**

2.3. Detection of MRSA by disc diffusion technique

Methicillin-resistance was determined by using the Kirby-Bauer disc diffusion method with 1 mg oxacillin. In 3 mL of sterile normal saline, three to five colonies were emulsified. The turbidity of the suspension was compared with the 0.5 McFarland turbidity standard and the suspension was inoculated on Mueller Hinton agar plates at 37^{O}C for 24 hours. An inhibition zone diameter of <=10 mm around the oxacillin disc was considered as resistant; 11-12 mm indicated intermediate, and >= 13 mm was considered sensitive. For the cefoxitin disc, an inhibition zone diameter of <=21 mm was considered as resistant and >= 22 mm was considered as sensitive **(CLSI.,2011).**

2.4. Antimicrobial susceptibility testing

To determine the susceptibility of the isolated *S. aureus* to different antibiotics, a standard agar-disc diffusion (Kirbey-Bauer) assay using Mueller Hinton agar (MHA) (HiMedia, India) plates was conducted (**Sharmin *et al*.,2014).**Asuspension of the test organism was prepared by adjusting the turbidity of the broth in phosphate buffer saline by comparing with that of the McFarland standard solution of 0.5 **(Bauer *et al*.,1968).** By means of a sterile cotton swab, a uniform lawn of bacterial growth was prepared on the MHA plates. A total of 20 antibiotic discs including methicillin (5 mg), tobramycin (10 mg), amikacin (30 mg,) imipenem (10 mg), azithromycin (15 mg), ceftriaxone (30 mg), cefoxitin (30 mg), cephradine (30 mg), vancomycin (30 mg), chloramphenicol (30 mg), tetracycline (30 mg),ciprofloxacin (5 mg), clindamycin (2 mg), , gentamycin (10 mg), netilmicin (30 mg), nitrofurantoin (300 mg), oxacillin (1 mg), penicillin (10 mg), trimethoprim/sulfamethoxazole (25 mg), and erythromycin (15 mg), were applied aseptically on the surface of the inoculated plates in an appropriate spatial arrangement

using a sterile needle. The plates were incubated at 37^{O}C for 12-18 hours and examined for zones of inhibition (mm) **(Munshi *et al*.,2012).**

2.5. Detection of VRSA by MIC of vancomycin

The minimum inhibitory concentration (MIC) of vancomycin (Oxoid, UK) was determined by the tube dilution method. MullereHinton Broth was prepared with 4-512 mg/mL of vancomycin. By using a direct colony suspension method, 0.5 McFarland equivalent bacterial inoculums that prepared in normal saline after culturing for 24 hours on an agar plate, Bacterial suspension were isolated on agar plates of the same media.emulsify 2 to 3 colony were emulsified in 0.5ml saline then 5 µL were spotted on brain heart infusion agar plates amended with 3 different conc of vancomycin (2µg/ml, 4µg/ml and 8µg/ml) according to CLSI **(CLSI, 2011).** The plates were incubated for 24 hours at 37C and checked for any visible growth.For detection of VRSA, an MIC of vancomycin >= 8mg/mL was considered resistant,<=2 mg/mL was sensitive, and 4-8 mg/mL was VISA **(CLSI.,2011).**

2.6. PCR detection of resistant and virulent genes

The PCR assay was performed in a total volume of 25 ul containing 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl2, 0.25mM each of deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 1 U of Taq DNA polymerase (Promega Corporation). DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 95^{o}C for 10 minutes, 33 cycles of amplification (denaturation at 95^{o}C for 45 seconds, annealing at 54^{o}C for 45 seconds, and extension at 72^{o}C for 1 minute), and a final extension at 72^{o}C for 10 minutes in a thermal cycler (Mastercycler gradient, Eppendorf AG). Analyze the PCR products were on 1.5% agarose gel with 0.53 Tris-borate-EDTA buffer. A 100-bp DNA ladder (Promega Corporation) was used as the molecular size marker. The gels were stained with 1% ethidium bromide and visualized under UV light **(Saha *et al.*,2008).**

3-Results

3.1-Isolation and identification of *staphylococcus aureus* from different clinical samples

Our results observed in table (1), there was 348 (67.71 %) isolates show β-hemolytic feature on blood agar plates, about 346 isolates (67.32 %) give a +ve results for growth on mannitol salt agar, only308 isolates (59.93 %) show mannitol fermentation.
Table (1): Presumptive identification of clinical staphylococcus samples isolated from examined clinical samples.

514 clinical sample	Number	%
B-hemolysis	348	67.71
Growth on mannitol salt agar	346	67.32
Mannitol fermentation	308	59.53
Negative	128	24.9

3.2-Identification of isolated strain using microscopical and biochemical reactions:

From our results observed in table (2) show that, about 296 (96.1 %) isolates were confirmed as *staphylococcus aureus* based on the positive results with coagulase test, slide agglutination test, tube agglutination test and DNAse test.The results also cleared that, there was 12 samples (2.38 %) classified as G+ve cocci that gave a variable results with catalase test, and negative results with slide agglutination test, tubeagglutination test and DNAse test.

Table (2): Microscopical and biochemical reactions that used for detection of *Staphylococcus aureus*.

308 isolates	microscopic examination	catalase test	coagulase test		DNAse test
			Slide coagulase Test	Tube coagulase Test	
296 (96.1 %)	Gm +ve cocci	+	+	+	+
12 (3.89 %)	Gm +ve cocci	variable	_	_	_

3.3. Phenotypic detection of methicillin resistance among *Staphylococcus aureus* isolates:

3.3.1. Disk diffusion method:

All strains proved to be *Staphylococcus aureus* in this study were examined for methicillin resistance by disk diffusion methods; the result revealed the presence of 215(72.6%) resistant isolates, 32(10.8%) intermediate sensitive isolates and 49(16.5%) sensitive isolates.

Table (3): Disk diffusion method for detection of MRSA.

Type of *Staphylococcus aureus* isolates	Number	(%)
MSSA	49	16.5
MISA	32	10.8
MRSA	215	72.6
Total number of isolates	269	100

3.4-Detection of vancomycin resistant *staphylococcus aureus* (VRSA) isolates.

In our results observed in table (4), the higher incidences of Staphylococci observed in VSSA that reached to 184 (85.58 %) followed by VISA 19 (8.8 %), followed by VRSA 12 (5.5 %).

Table (4): Types of vancomycin susceptible staphylococci.

Type of *Staphylococcus aureus* isolates	Number	(%)
VSSA	184	85.58
VISA	19	8.8
VRSA	12	5.5
Total number of isolates	215	100

3.5-Factors affecting Incidences of VRSA among examined clinical samples:

3.5.1-studyarea :

From our results observed in table (5) observe that, the higher incidences observed in Beni Swef hospital samples that reached to 4 (33.34 %), Asuit hospital 4 (33.34 %)Demerdash Hospital 3 (25 %) and the lower incidences observed in S. Galal Hospital 1 (8.32).

Table (5): Incidences of VRSA among samples collected from different localities

Hospital	Number of isolets	(%)
Beni Swef Hospital	4	33.34
Assuit Hospital	4	33.34
Demerdash Hospital	3	25
S. Galal Hospital	1	8.32
Total number of isolets	12	100

3.5.2-Type of specimen:

Our results observed in table (6), the incidences VRSAdiffer according to the type of the collected sample of either sputum, pus, blood, swab and urine. The higher incidences observed in sputum 3 (25 %), pus 3 (25 %) blood 2 (16.68 %) swab 2 (16.68 %) and the lower incidences observed in urine 1 (8.32 %).

Table (6): Clinical samples which gave positive VRSA

Type of specimen	Number of strain	%
Sputum	3	25
Pus	3	25
Blood	2	16.68
Swab	2	16.68
Urine	1	8.32
Total number of strain	12	100

3.5.3-Hospitalized and non hospitalized

Our results observed in table (7) cleared that, the incidences of VRSA reached to 6 (50 %) in hospitalized samples and 6 (50 %) in non-hospitalized sample.

Table (7): Samples that gave positive VRSA

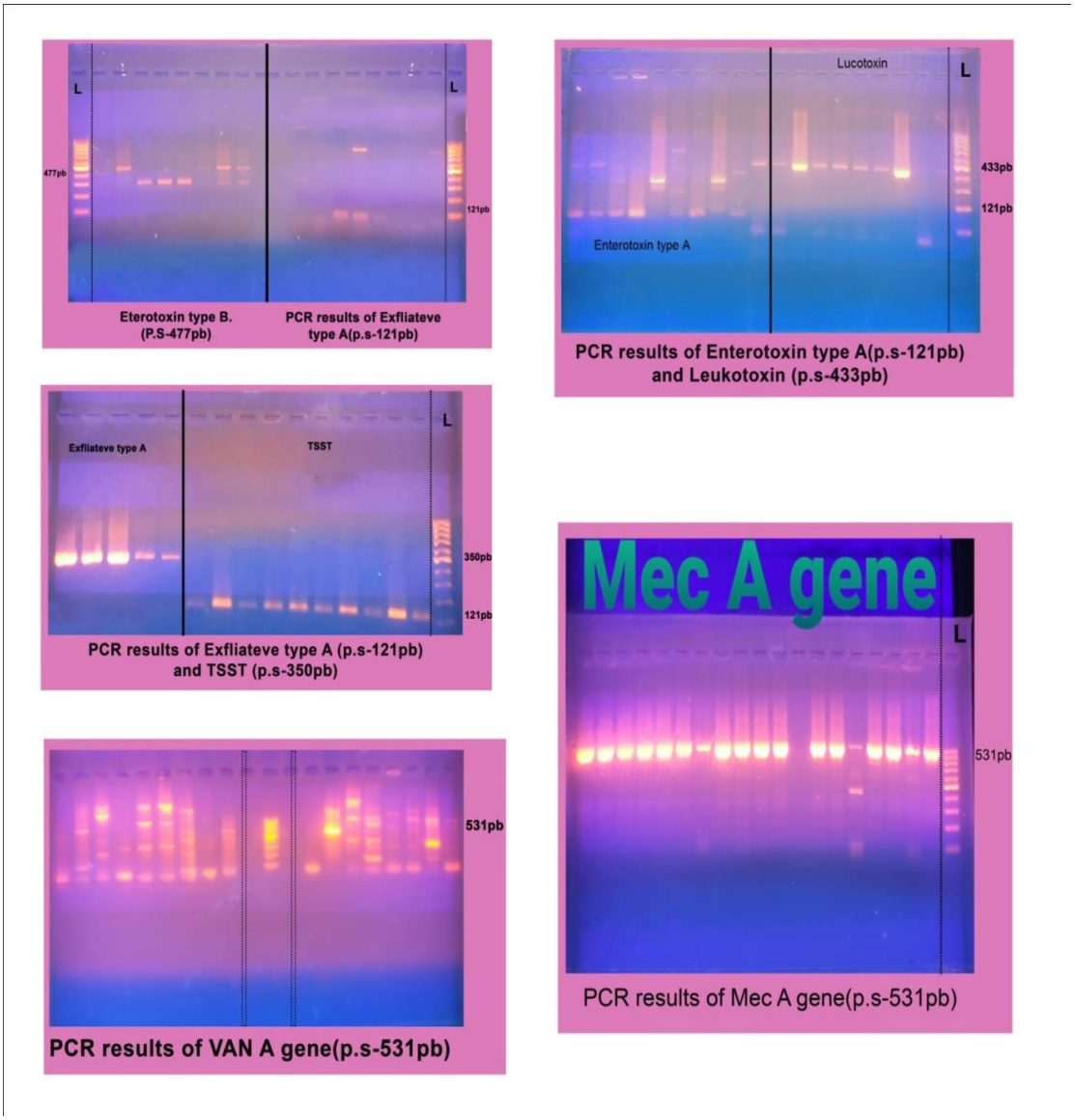
Hospitalized	Number of strain	%
Hospitalized	6	50
Non-Hospitalized	6	50
Total number of strain	12	100

3.5.4- Genes responsible for antibiotic resistance:

Our results observed in table (8), the resistant genes detected from 31 strain (VISA and (VRSA) were Mec A 15 (48.3 %),Van A 12(38.7%), Panton valentine leucocidin toxin (leukotoxin) 16 (51.6 %), Enterotoxin type A 18 (58 %) followed by TSST 14 (45.1 %), and the lower incidences observed in genes of Exfoliative toxin type A 5 (16.1 %) and Exfoliative toxin type B 1 (3.22 %).

Table (8): Incidences of different virulence genes responsible for resistance of VRSA

Gene type	Number of strains examined	Product size (bp)	Positive samples	
			Number	%
Panton valentine leucocidin toxin (leukotoxin)	31	433bp	16	51.6
Enterotoxin type A	31	121bp	18	58
Toxic shock syndrome toxin (TSST)	31	350bp	14	45.1
Exfoliative toxin type A	31	165bp	5	16.1
Enterotoxin type B	31	477bp	1	3.22
methicillin-resistant gene type A (Mec A)	31	531bp	15	48.3
Vancomycin-resistant gene type A (Van A)	31	531bp	12	38.7



(Figure No.1) PCR amplification of different resistant genes responsible for virulence and antibiotic resistance.

4-DISCUSSION

Antimicrobial resistance (AMR) whichremains a global setback in controlling common bacterial infectious diseases in both the resource-limited and developed countries **(Hart *et al*.,1998).** The current drift in the progression in AMR and drug development could lead to a "post-antibiotic era" with more than 4 million deaths projected per year by 2050, in Asia zone this indication from WHO**(World Health Organization. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018; O'Neill *et al*.,2014).** In our work we found that the frequency of VRSA infection has been increased. A majority of the studies in this analysis included data from both inpatients(hospitalized) and outpatients (non- hospitalized). Therefore, we assume our data represent a combination of community-acquired (CA) and hospital-acquired (HA) infections **(Song *et al.,2011).***Staphylococcus aureus* is the most

dangerous human pathogen among Staphylococci genus, the clinical significance of *Staphylococcus aureus* is largely due to the ubiquity of this species. *Staphylococcus aureus* is found on wide variety of habitats and is frequently part of the normal human micro flora; this species are commensals on human skin, and the vagina**(Kloos *et al*.,1999).**Wide range of illnessescan be caused by*Staphylococcus aureus* from minor skin infection, such as boils, carbuncles, furuncle, impetigo, scalded skin syndrome and abscess, to life threatening diseases such as pneumonia, osteomyelitis, meningitis, endocarditis, toxic shock syndrome and septicemia. Its incidence is from skin, soft tissues, bone, respiratory, joint, endovascular to wound infections**(Kluytmans *et al*.,1997).***Staphylococcus aureus* become methicillin resistant by the acquisition of the *mecA* gene which encode a penicillin-binding protein (PBP2a) with low affinity for β-lactam **(Chambers.,1997).**

Among this study, the percentages of VRSA isolated from clinical samples were as follow in sputum 3 (25 %), pus 3 (25 %) blood 2 (16.68 %) swab 2 (16.68 %) and the lower incidences observed in urine 1 (8.32 %). This result is confirmed by **(Grundmann *et al*.,2005).** who reported that screening of patients by obtaining culture specimen from body site such as the anterior nares (nasal passage) alone will identify 80% of those patients harboring VRSA, and screening from additional sites will increase the sensitivity to 92%. The incidences of VRSA reached to 6 (50 %) in hospitalized samples and 6 (50 %) in non-hospitalized sample.This result is proved by **(Cong *et al*.,2020).**who reported that VRSA infection are mainly confined to (50 %) in hospitalized samples and (50 %) in non-hospitalized sample.There are About 296 (96.1 %) isolates were confirmed as *staphylococcus aureus* based on the positive results with coagulase test, slide agglutination test, tube agglutination test and DNAse test. The results also cleared that, there was 12 samples (2.38 %) classified as G+ve cocci that gave a variable results with catalase test, and negative results with slide agglutination test, tube agglutination test and DNAse test. The current results are in agreement with (**Shariati Aet al.,2020)**who report that the presence of coagulase-negative VRSA in intensive care unit. Disk diffusion method detect all strains proved to be Staphylococcus aureus in this study were examined for methicillin resistance by disk diffusion methods; the result revealed the presence of 215 resistant isolates, 32 intermediate sensitive isolates and 49 sensitive isolates. Similar results were obtained by **(Daviedet *et al*.,2044).**who reported that cefoxitin disks are prior to oxacillin disks as it has 100% sensitivity, as cefoxitin induce greater production of PBP2a so it is a good assay for detection of low level MRSA. In the present study our results observed that, The higher incidences of Staphylococci observed in VSSA that reached to 184 (85.58 %) followed by VISA 19 (8.8 %), followed by VRSA 12 (5.5 %).The present results are in agreement with (**Shariati *et al*.,2020).**who report that Global prevalence and distribution of vancomycin resistant, vancomycin intermediate and heterogeneously vancomycin intermediate Staphylococcus aureus clinical isolates.In the present study our results observed that, the higher incidences observed in Beni Swef Hospital samples that reached to 4 (33.34 %), Asuit Hospital 4 (33.34 %),Demerdash Hospital 3 (25 %) and the lower incidences observed in S. Galal Hospital 1 (8.32). Similar results were obtained by **(Abd El-Aziz *et al*.,2018).**who report that near different percentage from different hospitals. In our study, there are the resistant genes detected from 31 strain (VISA and VRSA) were Mec A 15 (48.3 %),Van A 12(38.7%), Panton valentine leucocidin toxin (leukotoxin) 16 (51.6 %), Enterotoxin type A 18 (58 %) followed by TSST 14 (45.1 %), and the lower incidences observed in genes of Exfoliative

toxin type A 5 (16.1 %) and Exfoliative toxin type B 1 (3.22 %). Our results are in agreement with **(Islam *et al*.,2015).**who report that Fifteen of the 44 isolated strains of *S. aureus* (34.09%) were MRSA as identified by the cefoxitin disc diffusion method and the MIC of oxacillin, and all of them were *mecA* positive on PCR. Fourteen (31.82%) strains were resistant to methicillin based on the oxacillin disc diffusion method. Four (26.67%) of the 15 *mecA* positive strains were also positive for the PVL gene.

5-CONCLUSION

Our study concluded that *Staphylococcus aureus* especially methicillin-resistant *S. aureus* (MRSA), is emerging as a major public health problem in hospital and community settings, causing a many of diseases.The concentration of *staphylococcus aureus* in the clinical sample has been increased and this type of bacteria had become wide disseminated that is almost methicillin-resistant *S. aureus* (MRSA), and a lot of them become VISA and VRSA that have a lot of virulence factors, therefore consider a danger and threat to human life, and for us it is considered an epidemic that we are afraid of in the future, and it will be difficult to treat. Therefore, we need a new treatment strategy. We need a proper diagnosis. We need to reduce the use of the empirical therapy.

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انتشار المكورات العنقودية الذهبية المقاومة للفانكومايسين (VRSA) في بعض المستشفيات المصرية

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تم تجميع سلالات المكورات العنقودية الذهبية من ما يقرب من514 عينة سريرية تم جمعها من المرضى الذين تم قبولهم في مستشفيات بني سويف العامة ومستشفي جامعة أسيوط والمستشفى الجامعي ببني سويف,ومستشفى الدمرداش ومستشفى سيد جلال.

وتم قياس مقاومة الفانكومايسين بطريقة تخفيف المرق.وباستخدام طريقة الانتشار القرصي تم تحديد المقاومه ضد المضادات لحيويه المختلفه. كما تم إجراء فحص الجينات المقاومة والجينات الخبيثه بطريقة تفاعل البلمره المتسلسل ، ومن 514 عينة سريرية وجدنا أن عدد سلالات المكورات العنقودية كانت 308 سلالة (59.9٪) ، ووجدنا أن عدد سلالات المكورات العنقودية الذهبية كانت 296 سلالة (96.1٪). كانت سلالة المكورات العنقودية الذهبية المقاومة للميثيسيلين 215 (72.6٪). تم تقسيم سلالات MRSA المجمعة الي 184 سلاله VSSA(85.5٪) ، و 19سلاله VISA(8.8٪)) ، و 12 سلالة VRSA(5.5٪) .وقد تم رصد الجينات المقاومة من 31 سلالة (VISA و VRSA) كانت علي النحو التالي :

Mec A في 15 عزلة (48.3٪) ، Van A في 12 عزلة (38.7٪) ، Panton valentine lucocidineoxinlucotoxin) في 16 عزلة (51.6٪) ، و سم معوي من النوع A في 18 عزلة (58٪) تليها TSST في 14 عزلة (45.1٪) ، وقد لوحظت ان الاقل تواجدا في جينات التوكسين المقشر من النوع A في 5 عزلات (16.1٪) والسم التقشري من النوع B في عزلة واحدة (3.22٪) ،

وقد أظهرت نتائج الدراسة أن معدل الانتشار لل VRSA مرتفع في مصر ، وضرورة اكتشاف الأدوية الجديدة والفعالة ضد VRSA.

الكلمات المفتاحية : المقورات العنقودية الذهبية , المقورات العنقودية الذهبية المقاومة للميثيثلي , طرق تخفيف المرق , المقورات العنقودية الذهبية المقاومة للفانكومايثن , المقورات العنقودية الذهبية الحساسة للفانكومايثن