بسم الله الرحمن الرحيم

Shendi University

College of Graduate Studies and Scientific Research

Detection and Molecular Characterization of Methicillin and Vancomycin Resistant *Staphylococcus aureus* and Sequencing of *mecA* and *arcC* Genes among Different Clinical Isolates from Shendi City

A thesis Submitted in Fulfillment of the Requirement for the Degree of PhD in Medical Laboratory Sciences (Medical Microbiology)

By

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الآية

(قُلْ لَوْ كَانَ الْبَحْرُ مِدَادًا لِكَلِمَاتِ رَبِّي لَنَفِدَ الْبَحْرُ قَبْلَ أَنْ تَنْفَدَ كَلِمَاتُ رَبِّي وَلَوْ جِئْنَا بِمِثْلِهِ مَدَدًا (109) قُلْ إِنَّمَا أَنَا بَشَرٌ مِثْلُكُمْ يُوحَى إِلَيَّ أَنَّمَا إِلَهُكُمْ إِلَهٌ وَاحِدٌ فَمَنْ كَانَ يَرْجُوا لِقَاءَ رَبِّهِ فَلْيَعْمَلْ عَمَلًا صَالِحًا وَلَا يُشْرِكْ بِعِبَادَةِ رَبِّهِ أَحَدًا (110)

صدق الله العظيم

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سورةالكهف

Dedication

This Project is dedicated to

the sole of My Father and My Mother…….

Who gave me the meaning of the life

My Lovely husband and Daughter

My sisters and My brothers

My friends and My colleagues……..

The persons whom I love, respect and appreciate…..

To all who has ever taught me anything

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List of Abbreviations

Abbreviations	Explanation
CA-MRSA	Community-Acquired MRSA
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase Negative Staphylococci
HA-MRSA	Hospital-Acquired MRSA
HVR	Hyper-Variable Region
MHC	Major Histo Compatibility
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MRSA	Methicilin Resistant *Staph. aureus*
NAG	N-acetylglucosamine
NAM	N-acetylmuramic
NCCLs	National Committee for Clinical Laboratory standard
PBPs	Penicillin-Binding Proteins
PVL	Panton-Valentine Leukocidin
SCCmec	Staphylococcal Cassette Chromosome mec
SSSS	*Staphylococcal* scalded-skin syndrome
TMP-SMX	Trimethoprime, sulfamethoxazole
TSS	Toxic Shock Syndrome
TSST	Toxic Shock Syndrome Toxin
VISA	Vancomycin-Intermediate *Staph. aureus*
VRSA	Vancomycin-Resistant *Staph. aureus*

ABSTRACT

Background: An increased prevalence of MDR phenomenon has been observed worldwide. Among the most threatening antibiotic-resistant pathogens known are MRSA. In many countries, the situation appears dusky due to abuse of antibiotics and lacking of regulations towards controlling the emergence of infectious diseases.

Objective: The present study aimed to determine the frequency of methicillin and vancomycin resistant *Staph. aureus* and also it aimed to detect the presence of *mec*A*gene* in MRSA and *van*A and *van*B by PCR and correlate the results with the conventional methods.

Methodology: Three hundred (*n*= 300), clinical specimens were collected from patients with different diseases from various hospitals at Shendi City, Northern Sudan in the period from October 2012 to September 2013. *S. aureus* conventional methods and PCR were used for identification scheme. The *S. aureus* were analyzed for their susceptibility to different antibiotics using disk diffusion method. All MRSA isolates were subjected to PCR to amplify *mec*A gene, while VRSA were tested for the presence of *van*A and vanB gene.

Results: Of the total 300 clinical specimens (200) were confirmed as *S. aureus* (66.7%) among which 123(61.5%) were identified as MRSA, 58.5% from MRSA was detect *mec*A gene by PCR, while 41.5% of MRSA strains were *mec*A gene negative. While all MSSA showed negative results for *mec*A gene. Eight out of the 123 MRSA isolates (6.5%) were identified as VRSA when using conventional DST method, *van*B was detected among only 3 VRSA isolates (37.5%) while *van*A was not detected in any isolates.

All isolated genes were subjected to DNA sequencing, the sequence alignment showed that no mutation was carried or detected in the *mec*A gene or in *arc*C genes.

Conclusions: The study concluded that while amplification of *mec*A gene failed to detect all MRSA strains in the community, it is highly recommended to search for other intrinsic factors that may compete *mecA*gene in producing similar phenomenon, also it concluded that PCR assay was rapid and accurate technique for the identification of *van*B gene of VRSA strains as compared to the conventional methods since the time was taken is less and can help efficiently in controlling and management of the emergence of multi drugs resistant pathogen such as *S. aureus*.

الملخص

الملخص: لوحظ فى جميع أنحاء العالم ظاهرة زيادة إنتشار المقاومة للمضادات الحيوية المعروفة فى الفترة الاخيرة وهذا الوضع الداكن ينتج من سوء إستخدام المضادات الحيوية وتفتقرالى لوائح نحو السيطرة على معالجة الأمراض المعدية.

الهدف: تهدف هذه الدراسة الى تحديد وتيرة المضاد الحيوى الميثسلينوالفانكوميسين المقاوم للبكتيريا الكروية العنقودية الذهبية وإنتاجها الى جينات *mec*A ,*van*A and *van*B .

وتهدف أيضاً للمقارنة بين الطريقة التقليدية وطريقة تفاعل البلمرة المتسلسل الطريقة الحديثة للكشف عن الجينات المعزولة من البكتيريا الكروية العنقودية

المنهجية: تم جمع300 عينة من العينات السريرية من المرضى الذين يعانون من أمراض مختلفة من مختلف مستشفيات مدينة شندى بولاية نهر النيل فى شمال السودان فى الفترة من أكتوبر2012 الى سبتمبر2013 .تم عزل البكتيريا الكروية العنقودية الذهبية بالطريقة التقليدية وقد حللت لمعرفة قابليتها للمضادات الحيوية الميثسيلينوالفانكوميسين بإستخدام طريقة نشر القرص .وإستخدام الطريقة الحديثة طريقة تفاعل البلمرة المتسلسل للكشف عن الجينات *mec*A, *va*nA and *van*B.

النتيجة: من بين300 كانت 200 عينة بكتيريا عنقودية ذهبية (66.7٪) منها 123تم تحديدها بإعتبارها مقاومة للمثيسلين (61.5٪). (58.5%) كانت نسبة العنقودات الكروية المقاومة للميثسلين التى تحتوى على *mec*A جين وذلك عن طريق تفاعل البلمرة المتسلسل و (41.5٪) منها سلالات سلبية لاتحتوى على *mec*A جين . وكانت ثمانية من عدد 123العزلات المقاومة للميثسلين (6.5٪) تم تحديدها بإعتبارها مقاومة للفانكوميسين عند إستخدام الطريقة التقليدية. وعند إستخدام الطرق الحديثة تم الكشف عن 3 فقط (37.5٪) تحتوى على *van*B، في حين لم يتم الكشف عن *van*A فى أي من العزلات.

تعرضت جميع الجينات المعزولة لتسلسل الحمض النووي، وأظهرت محاذاة تسلسل أنه ليس هناك طفرات حدثت فى الجينات المحتوية على *mec*A وكذلك لم تحدث فى *arc*C.

الإستنتاجات: خلصت الدراسة إلى أنه في حين فشل التضاعف فى جين *mec*A للبكتيريا العنقودية المقاومة للميثسيلين في المجتمع، ويوصى للبحث عن العوامل الجوهرية الأخرى التي قد تنافس الجينات في إنتاج ظاهرة مماثلة، وأن طريقة تفاعل البلمرة المتسلسل تقنية دقيقة للتحديد الجيني للسلالات بالمقارنة مع الطرق التقليدية وأكثر حساسية وتخصصية, وإستخدام السلالات الجينية

*van*B لتساعد بشكل فعال في السيطرة وإدارة ظهورعقاقيرمتعددة للبكتريات الممرضة المقاومة للمضادات الحيوية مثل بكتريا المكورة العنقودية الذهبية

CHAPTER ONE

Introduction and Objectives

1. Introduction

Staphylococcus aureus (*Staph. aureus*) is a major cause of potentially life-threatening infection acquired in health care and community setting (Linda *et al.*, 2006).

It is very successful hospital and community acquired pathogen it causes abroad spectrum of diseases from mild skin infection to more serious infection include septicemia, pneumonia, endocarditis, osteomylitis and wound infection. Pathogenicity is related to number of virulence factors that allow it to adhere to any surface, invade or avoid the immune system and cause harmful toxic effect to the host. These factors include cell surface and exoprotein (exotoxin, exofoliatins, toxic shock syndrome toxin (TSST) and panton valentine leucocidin pvl, *Staph. aureus* has ability for posing challenge to resist most antibiotics (Sajna *et al*., 1999).

Bacteria can sometime adapt to the antibiotics used to kill them, this adaptation which can involve structural changes or the production of enzymes that render the antibiotic useless, can make the particular bacterial species resistant to the particular antibiotic, these occur in two ways, the first methods is known as inherent or natural resistance and the second category of adaptive resistance is called acquired resistance. The resistance is almost always due to change in genetic make-up of the bacterial genome. Antibiotic resistance is a problem that develops when antibiotics are overused or misused. If an antibiotic is used properly to treat an infection, then all the infectious bacteria should be killed directly, or weakened such that the host is immune response will kill them. These surviving bacteria have demonstrated resistance. If the resistance is governed by a genetic alteration, the

genetic change may be passed on to subsequent generation of bacteria (Lerner and Lerner Wilmoth 2003). *Staph. aureus* resists penicillin by producing enzyme penicillinase which destroy β-lactam ring in penicillin structure (Greenwood, 2002).

Staph. aureus infection can be treated with antibiotics such as methicillin a type of penicillin (Greenwood, 2002).

Staph. aureus has long been recognized as a major pathogen of hospital - acquired infections. Over the last decade, methicillin resistant *Staph. aureus (*MRSA) strains have become endemic in hospitals worldwide. In addition, it is now incipient community pathogen in many geographical regions (Lowy, 1998).

MRSA is important because, in addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotic, with the exception of glycopeptide antibiotics (Chambers, 1997; Brumfil and Hamilton, 1989). In 1980s, because of widespread occurrence of MRSA, empiric therapy for staphylococcal infections (particularly nosocomial sepsis) was changed to vancomycin in many health care institutions. Vancomycin use in United States also increased during this period because of the growing numbers of infections with *Clostridium difficile* and coagulase negative staphylococci (CoNS) in health care institutions (Ena et *al.*, 1993; Cunha, 1995), thus, the early 1990s have shown a discernible increase in vancomycin use. As a consequence, selective pressure was established that eventually lead to the emergence of strains of *Staph. aureus* and other species of *Staphylococcus* with decreased susceptibility to vancomycin and other glycopeptides (Tenover *et al.*, 2001).

In 1997, the first strain of *Staph. aureus* with reduced susceptibility to vancomycin and teicoplanin was reported from Japan (Hiramatsu *et al*., 1997). Shortly after, two additional cases were reported from United States (Centers for Disease Control and Prevention, 1997). However, first clinical isolate of vancomycin resistant

Staph. aureus (VRSA) was reported from United States in 2002 (Centers for Disease Control and Prevention, 2002). More recently some workers have reported vancomycin resistant staphylococcal stains from Brazil (Palazzo *et al.*, 2005), and Jordan (Bataineh, 2006).

Methicillin resistance *Staph. aureus* (MRSA) is phenotypically associated with the presence of altered penicillin binding protein PBP_2a (Sajna *et al.,* 1999). Some strains of *Staph*-like MRSA have become resistant to antibiotic that once destroyed it. MRSA was first discovered in 1961. It is now resistant to methicillin – amoxicillin-penicillin-oxacillin –vancomycin and other antibiotics (Gerand J *et al*., 2004)

Methicillin resistance in MRSA is coded by *mecA* genes, which is carried in mobile genetic element termed the *Staphylococcal* cassete chromosome *mec* SCC*mec* (Susan *et al.,* 2006). The increased prevelance of methicillin resistance in *Staph* species has led to wide spread use of glycopeptides antibiotics such as vancomycin (Michigan *et al.,* 2007).

Vancomycin continues to be used as a first-line antimicrobial agent for the treatment of infection with MRSA, the action of vancomycin it binds with peptide chain inhibiting trans-peptidation of amino acid (Hakim *et al.,* 2007).

Because alternative treatments are limited, development of resistance to vancomycin can make treatment of MRSA infections increasingly difficult.

But recently found resistance to vancomycin in *Staph. aureus* (VRSA) although vancomycin resistance was first reported for enterococci in 1988 (Vancomycin resistant *Enterococcus* spp (Sajna *et al.*, 1999).

VRSA was initially isolated in Michigan Hospitals. Confirmatory identification by conventional biochemical methods and antimicrobial drug susceptibility testing were performed by MDCH's Bureau of laboratories, hence vancomycin resistance is defined as MIC (greater than or equal to 16 mg/ml). Then the isolates were

submitted to the center for disease control and prevention (CDC) (Michigan *et al.*, 2007).

The first clinical isolate of high level of vancomycine resistance *Staph. aureus* VRSA was not isolated until June/2002 [Michigan, MIC = 16mg/ml] this was closely followed by another VRSA isolate in Pennsylvania in September/ 2002 [MIC = 32mg/ml] these isolates were associated with chronic skin ulcer (Linda *et al.*, 2006).

Genetic exchange of antimicrobial resistance determinants among enterococci and *Staphylococci*. The resistance gene are typically found on conjugative plasmids or transposes one requirement for conjugative transfer of mobile genetic element is cell to cell contact between donor and recipient (Linda *et al.*, 2006). Also glycopeptides - resistant enterococcal phenotypes *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G have been described (Patel *et al.*, 2006).

The presence of the *van*A gene was confirmed by polymerase chain reaction and was located on a 60 kb plasmid. The DNA sequence of VRSA *van*A gene was identical to that of vancomycin resistance strain of *Enterococcus faecalis* in study of isolate form catheter tip in Michigan (Patel *et al.*, 2006).

1.2 Rationale

Based on the misuse of antibiotics in less developed societies such as those in Sudan, the thing that encourages the emergence of drug-resistant strains at all levels.

The recent emergence of MRSA in community associated infections highlight the success of this species as a pathogen and its ability to evade from the action of empirical antimicrobial agents.

Glycopeptides such as vancomycin provide effective therapy against most multidrug-resistant strains of *Staph. aureus.*

However, after an increase in the infection by vancomycin resistant and methicillin resistant *Staph. aureus* and the presence of resistance for several other antibiotics, detection of *van*A gene in VRSA and *mec*A gene in MRSA is important for the appropriate diagnosis and control of the infection.

Show the importance of MRSA in Sudan in (Shendi) compare with other result as (Maimona *et al.,* 2014) and Omar (2014) reports also Elimam *et al.,* (2014) reports.

1.3 Research Questions

1. Is there is a significant presence of MRSA in Shendi City?
2. Does vancomycin resistant *Staph. aureus* (VRSA) exist in Shendi City?
3. Which van gene (*van* A or *van* B) is responsible for the resistance phenomenon in Shendi?
4. Is there any detectable mutation (s) in any of the study genes?

1.4 Objectives

1.4.1 General Objective

The present study aimed to detect the frequency of MRSA and VRSA among patients with different clinical diseases and medical staff in Shendi City.

1.4.2 Specific Objectives

1. To isolate and characterize Staph. *aureus* from different clinical specimens in patients from Shendi City.
2. To determine antimicrobial profile of *Staph. aureus* against different antibiotics.
3. To use *mec*A gene as diagnostic tool for MRSA.
4. To validate the use *van*A and *van*B genes in the diagnosis of VRSA.
5. To sequence *arc*C, *mec*A, *van*A and *van*B and detect the presence of mutation.

CHAPTER TWO

2. Literature Review

2.1The Genus *Staphylococcus*

2.1.1 Definition

Genous *Staphylococcus* is a Gram-positive coccus that forms cluster, produces catalase, has an appropriate cell wall structure (including peptidoglycan type and teichoic acid presence). *Staphylococcus* species can be differentiated from other aerobic and facultative anaerobic Gram positive cocci by several simple tests. *Staphylococci* are facultative anaerobes (capable of growth both aerobically and anaerobically). All species grow in the presence of bile salts and all are catalase positive. Growth also occurs in a 6.5% NaCl solution. On Baird Parker Medium *Staphylococcus* spp. grow fermentatively, except for *Staph. saprophyticus*, which grows oxidatively. *Staphylococcus* spp. are resistant to bacitracin and susceptible to furazolidone (100μg disc: resistance = <15mm zone of inhibition). Further biochemical testing is needed to identify down to the species level (Mackie and McCartney, 2000).

One of the most important phenotypical features used in the classification of Staphylococci is their ability to produce coagulase, an enzyme that causes blood clot formation. *Staph. edius, Staph. lutrae, pseudintermedius* and *Staph. schleiferi* subsp. coagulans. These species belong to two separate groups - the *Staph. aureus* (*Staph. aureus* alone) group and the *Staph. hyicus-intermedius* group(Mackie and McCartney, 2000).

Staph. aureus is an important nosocomial and community-acquired pathogen. *Staph. aureus* is the most common cause of hospital-acquired infection, causing clinical disease in 2% of all patient admissions in the UK. Not only does it cause enormous numbers of infections, but *Staph. aureus* in hospitals are becoming

increasingly resistant to antibiotics. In several industrialised nations including parts of Europe, the US and Japan, 40-60% of all hospital *Staph. aureus* are now resistant to methicillin (methicillin-resistant *Staph. aureus*, MRSA) (Greenwood *et al.*, 2002).

2.1.2 Historical Background

Staphylococcus was first discovered by Sir Alexander ogoston, Scottish surgeon, in 1880. He showed that a number of human pyogenic diseases were associated with a cluster-forming micro-organism. He introduced the *Staphylococcus* (Greek: staphyle=bunch of grapes; kokkos=grain or berry), now used as genus name for a group of facultatively anaerobic, catalase positive, gram positive-cocci (Greenwood *et al.*, 2007).

2.1.3 Classification

All Micrococcaceae family includes *Staphylococcus* family and *Micrococcus* produce spherical, Gram-positive cells (Betty *et al.*, 2007a). The genus *Staphylococcus* has at least 35 species; the three main species of clinical importance are *Staph. aureus, Staph. epidermidis* and *Staph. saprophyticus* (Brooks *et al.*, 2007). *Staph. aureus* differentiate from the other species in the genus by co-agulase test (Cheesbrough , 2007).

2.1.4 General Properties

Staph. aureus habitat is normal flora of human anterior nares, nasopharynx, perineal area and skin, it can colonize various epithelial or mucosal surfaces (Betty *et al.*, 2007a). *Staph. aureus* are relatively resistant to drying, heat and they withstand 50degree centigrade for 30min but are readily inhibited by certain chemicals for example 3%hexachlorophene (Brooks *et al.*, 2007). *Staph. aureus* is able to grow on agar containing 70-100g/L sodium chloride, ferment mannitol (Cheesbrough, 2007). *Staph. aureus* slowly ferments many carbohydrates, producing lactic acid but not gas, proteolytic activity varies greatly from one strain

to another. *Staph. aureus* characterized by producing pigment, best at room temperature (20-25degree centigrade), *Staph. aureus* usually form gery to deep yellow colonies and many colonies develop pigment upon prolong incubation, no pigment is produce anaerobically or in broth (Brooks *et al.,* 2007).

2.1.5 Toxins and Enzymes

Staphylococci can produce disease through both their ability to multiply and spread widely in tissues, and through their production of many extracellular substances, *Staph. aureus* produce catalase enzyme which convert hydrogen peroxide into water and oxygen. Also produce co-agulase,an enzyme-like protein that clot oxalated or citrated plasma, coagulase binds to prothrombin, together they become enzymatically active and initiate fibrin polymerization, co-agulase may deposit fibrin on the surface of *Staphylococci*, perphaps altering their ingestion by phagocytic cells, or their destruction within such cells (Brooks *et al*., 2007), Some strains produce saphyloxanthin-a carotenoid pigment that acts as a virulence factor, it has an antioxidant action that helps the microbe to evade killing with reactive oxygen used by the host immune system. It is thought that staphyloxanthin is responsible for *Staph. aureus* characteristic golden colour (Clauditz *et al*., 2006).

Other enzymes include deoxyribonuclease (DNASe) that destroy deoxyribonucleic acid, Hyaluronidase that facilitates spread in the tissues by destroying hyaluronic acid a component of connective tissue, Lipase which breakdown fat ,fibrinolysins which digests fibrin, and β-lactamases (antibiotic, inactivating enzymes), that lead to penicillin resistance (Cheesbrough, 2007). Some *Staph. aureus* produce exoproteins, alpha-lysin, beta-lysin, gamma-lysin, zeta-lysin that cause impairment of membrane permeability, and cytotoxic effects on phagocytic and tissue cells (Greenwood *et al*., 2007). Leukocidin, this toxin of *Staph. aureus* has two components which can kill white blood cells of humans and rabbits, the two components act synergistically on the white blood cells membrane.

The toxin is an important virulence in community associated methicillin resistant *Staph. aurues* infections (Brooks *et al.*, 2007). The overcome of host response by *Staph. aureus* include chemotaxis inhibitory protein which inhibits migration and activation of neutrophis (Cheesbrough, 2007), *Staph. aureus* has exfoliative toxin which is two distinct protiens refer to as epidermolytic toxins of the same molecular weight, epidermolytic toxin A is chromoosomal gene product and is heat stable (resists boiling for 20min) Epidermolytic toxin B is plasmid-mediated and heat-labile. Epidermolytic toxins are responsible for *Staph. aureus* scalded skin syndrome by dissolving mucopolysaccharide matrix of the epidermis, this toxin is superantigens (Brooks *et al.*, 2007) .

Most *Staph. aureus* produce toxic shock syndrome toxin what is called (TSST-1), is the same as enterotoxion F. TSST-1 is superantigens that binds to MHC II molecules, yielding T cell stimulation, which promotes the protein manifestations of toxic shock syndrome, Most *Staph. aureus*-exfoliative toxin producing strains belong to phage group II (Greenwood *et al.*, 2007). The toxin is associated with fever, shock, and multisystem involvement, including a desquamative skin rash.The gene of TSST-1 is found in about 20% of *Staph. aureus* isolates (Brooks *et al.*, 2007).

Staph. aureus enterotoxin is multiple (A-E, G-I, K-M) enterotoxins, approximately 50% of *Staph. aureus* can produce one or more of them, the enterotoxins are superantigen and are heat-stable, that resistant to the action of gut enzymes, they are important cause of food poising followed by diarrhea, and vomiting. The exfoliative toxins, TSST-1 and the enterotoxin gene are on chromosomal element called a pathogenicity island. It interacts with accessory genetic elements (bacteriophages) to produce the toxins (Brooks *et al.*, 2007). Ability of *Staph. aureus* to biofilm production is another important virulence factor (Betty *et al.*, 2007a).

2. 2 Pathogenicity of *Staphylococcus aureus*

2.2.1 Virulence Factors

Microorganisms that successfully invade host tissues increase their numbers by producing a range of factors that enable them to survive the onslaught of innate and specific immunity and which are responsible for the development of clinical disease. *Staph. aureus* expresses a number of factors that have the potential to interfere with host defense mechanisms. However, strong evidence for a role in virulence of these factors is lacking (Cheesbrough, 2006; Foster, 2008).

2.2.1.1 Structural Components

The cellular structure of *Staph. aureus* is complex. Most strains have polysaccharide microcapsules. The cell wall of *S. aureus* is structurally similar to that of Group A streptococci: both have a carbohydrate antigen, a protein component, and a mucopeptide (Stevens, 1999).

2.2.1.1.1 Capsule

The majority of clinical isolates of *Staph. aureus* express a surface polysaccharide. This has been called a microcapsule because it can be visualized only by electron microscopy after antibody labeling. The function of the capsule is not clear. It may impede phagocytosis, but in in vitro tests this was only demonstrated in the absence of complement (Foster, 2008).

2.2.1.1.2 Peptidoglycan and Cytoplasmic Membrane

A complex web of cross-linked peptidoglycan outside the cytoplasmic membrane provides the cell with mechanical strength. It is particularly abundant in Gram-positives, when it also contains strands of teichoic and lipoteichoic acid (Cheesbrough, 2006).

2.2.1.1.3 TeichoicAcids

The carbohydrate antigen is a teichoic acid. Antibodies to teichoic acid can be detected in normal human serum, and elevated antibody titers are present in

patients with deep-seated staphylococcal infections. Teichoic acid has no established role in virulence, and antibodies to this carbohydrate are not protective (Stevens, 1999).

2.2.1.1.4 Protein A

Protein A is a surface protein of *Staph. aureus* which interacts with the Fc component rather than the Fab component of IgG. Protein A may be antiphagocytic, but its role in virulence has not been clearly established (Weigelt *et al*., 2007; Foster , 2008).

2.2.1.1.5 Leukocidin

Staph. aureus can express a toxin that specifically acts on polymorph nuclear leukocytes. Phagocytosis is an important defense against staphylococcal infection so leukocidin should be a virulence factor, which consists of two leukotoxic proteins that are capable of disrupting lysosomal membranes (Weigelt *et al.,* 2007; Foster, 2008).

2.2.1.2 Enzymes Production

Enzymes are very important molecules found in every cell. Enzymes generally act as catalysts that increase the speed or rate at which substances in a cell get converted into other substances. Without enzymes, some reactions would take place too slowly or might not take place at all, each enzyme has a different job and many enzymes must work together to keep an organism alive and healthy (fridkin *et al.,* 2003).

2.2.1.2.1 Coagulase

The enzyme coagulase causes plasma to clot, thus promoting the fibrin meshwork that contributes to abscess formation (Weigelt *et al.,* 2007). It is an extracellular protein which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. Coagulase is a

traditional marker for identifying *Staph. aureus* in the clinical microbiology laboratory. However, there is no evidence that it is a virulence factor, although it is reasonable to speculate that the bacteria could protect themselves from host defenses by causing localized clotting (Foster, 2008).

2.2.1.2.2 Catalase

In the liver, there are several enzymes that act on certain toxic or poisonous compounds by removing hydrogen atoms from the poisons and transferring them to oxygen molecules. This detoxifies the poison but it creates a new compound, hydrogen peroxide (H_2O_2) that is very active and can be harmful to the organism. Fortunately there is another enzyme in the liver that helps break down the peroxide into water and oxygen. This enzyme is known as catalase. The catalase enzyme reduces the substrate, peroxide, to water and oxygen (Cheesbrough, 2006).

2.2.1.2.3 Hyaluronidase

Hyaluronidase is an example of enzymes that help pathogens to spread. It is produced by *staphylococci*, helps organisms to spread through the body by breaking down the hyaluronic acid intercellular junctions of connective tissue, leading to cellulitis (Steven , 1999; Cheesbrough, 2006).

2.2.1.2.4 Lipases

Lipases are large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures. Bacterial lipases are mostly extracellular and are produced by submerged fermentation. Lipases are serine hydrolases and have highstability in organic solvents (Gupta *et al.*, 2004).

2.2.1.2.5 Nuclease

Although expression and secretion of an extra-cellular nuclease by *Staph. aureus* have been documented for a long time, the specific role of *Staph. aureus* nuclease in pathogenesis is poorly understood (Evelien *et al.*, 2010).

2.2.1.2.6 Staphylokinase

Many strains of *Staph. aureus* express a plasminogen activator called staphylokinase. Also known as fibrinolysin, this extracellular protein is produced by several strains of *Staph. aureus* and dissolves fibrin clots, which contributes to the spread of the organism. Acomplex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. As with coagulase there is no evidence that staphylokinase is a virulence factor, although it seems reasonable to imagine that localized fibrinolysis might aid in bacterial spreading (Foster, 2008).

2.2.1.2.7 Penicillinase

Another important enzyme is penicillinase. Because penicillinase has no role in pathogenicity, *staphylococci* that produce penicillinase are no more virulent than nonpenicillinase-producing strains. Nevertheless, this enzyme is clinically and epidemiologically important, because it hydrolyzes the beta-lactam ring of penicillin, thereby inactivating the molecule. The production of penicillinase is controlled by plasmids, or episomes, which are extrachromosomal DNA molecules that replicate during cell division (Weigelt *et al.,* 2007).

2.2.1.3 Toxins Production

2.2.1.3.1 Alpha Toxin

Of even greater interest are the nonenzymatic toxins produced by *Staph. aureus*. α-Toxin is a cytotoxin and the best characterized and most potent membrane-damaging toxin of *Staph. aureus* by producing pores in cell membranes, thereby altering their permeability and resulting in cell damage or death. α -Toxin damages red and white blood cells and activates platelets. They carry high affinity sites which allow toxin to bind at concentrations that are physiologically relevant (Weigelt *et al.,* 2007; Foster, 2008).

2.2.1.3.2 Beta Toxin

ß-toxin is a sphingomyelinase which damages membranes rich in this lipid. The classical test for ß-toxin is lysis of sheep erythrocytes. The majority of human isolates of *Staph. aureus* do not express ß-toxin. A lysogenic bacteriophage is inserted into the gene that encodes the toxin (Foster, 2008).

2.2.1.3.3 Delta Toxin

The δ-toxin is a very small peptide toxin produced by most strains of *S. aureus*. It is also produced by *Staph. epidermidis* and *Staph. lugdunensis*. The role of δ -toxin in disease is unknown (Foster, 2008).

2.2.1.3.4 ɤ-Toxin and Leukocidin

The ɤ-toxin and the leukocidins are two-component protein toxins that damage membranes of susceptible cells. The proteins are expressed separately but act together to damage membranes. The ɤ-toxin locus expresses three proteins. The B and C components form a leukotoxin with poor hemolytic activity, whereas the A and B components are hemolytic and weakly leukotoxic (Stevens *et al.*, 2007).

2.2.1.3.5 Panton-Valentine Leukocidin (PVL)

The classical Panton and Valentine (PV) leukocidin is distinct from the leukotoxin expressed by the ɤ-toxin locus. It has potent leukotoxicity and, in contrast to ɤ-toxin, is non-hemolytic. Only a small fraction of *Staph. aureus* isolates (2% in one survey) express the PV leukocidin, whereas 90% of those isolated from severe dermonecrotic lesions express this toxin. This suggests that PV leukocidin is an important factor in necrotizing skin infections (Stevens *et al.*, 2007).

2.2.1.3.6 Exfoliative Toxins

EF toxins are implicated in the disease staphylococcal scalded-skin syndrome (SSSS), which occurs most commonly in infants and young children. It also may occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS (Foster, 2008).

2.2.1.3.7 Superantigens: Enterotoxins and Toxic Shock Syndrome Toxin

Staph. aureus can express two different types of toxin with superantigen activity, enterotoxins, of which there are six serotypes (A, B, C, D, E and G) and toxic shock syndrome toxin (TSST-1). Enterotoxins cause diarrhea and vomiting when ingested and are responsible for staphylococcal food poisoning. When expressed systemically, enterotoxins can cause toxic shock syndrome (TSS) - indeed enterotoxins B and C cause 50% of non-menstrual TSS. TSST-1 is responsible for 75% of TSS, including all menstrual cases (Foster, 2008).

2.2.2 Transmission

Staph. aureus is normal flora, spread of patient's endogenous strain to normally sterile site by traumatic introduction, and may transmitted by fomites, air, or unwashed hands of health care workers, especially in the nosocomial setting. May be transmitted from infected skin lesion of health care worker to patient (Betty *et al.*, 2007b).

2.2.3 Pathogenesis

Diseases cause by *Staph. aureus* differ from local lesion to severe pneumonia, through their ability to multiply, spread in tissue and production of varying enzymes and exotoxins (Brooks *et al.*, 2007). *Staph. aureus* establish in hair follicle lead to tissue necrosis, surround by wall of coagulate fibrin. Healing include drainage of liquid center necrotic tissue. In abscess or focal suppuration, from any focus organisms may spread via the lymphatics and blood stream to other part of the body, suppuration within veins, associated with thrombosis is a common feature of dissemination (Brooks *et al.*, 2007). Blistering diseases due to epidermolytic toxin that induce intra-epidermal blisters at granular cell layer. Such blister range in severity from trivial to the distended blister of pemphigus neonatorum, the most manifestiation of epidermolytic toxin is the scalded skin

syndrome in small children where toxin spread systematically in individual who lack neutralizing antitoxin (Greenwood *et al.*, 2007).

In osteomyelitis, the primary focuses of *Staph. aureus* is a typically in terminal blood vessel of the metaphysis of a long bone, leading to necrosis bone and chronic suppuration (Brooks *et al.*, 2007). Other diseases include toxin shock syndrome due to TSST-1. A link was established with use of highly absorbant tampons in menstruating women, although non-mentrual cases are now as common (Greenwood *et al.*, 2007). *Staph. aureus* food-poisoning is caused by the ingestion of preformed toxin in contaminated food, often dairy products. Occasionally Staphylococcal enterocolitis is a complication of broad spectrum antibiotic therapy (Cheesbrough, 2007). *Staph. aureus* common cause of neonatal septicaemia, it found in blood within invasive disease, and common cause of acute end (Betty *et al.*, 2007b). The invasive nature of this organism always present a threat for deeper tissue invasive, bacteraemia, and spread to one or internal organ including the respiratory tract (Betty *et al.*, 2007b). Staphylococcal pneumonia is confluent bronchopneumonia that is often unilateral or more prominent on one side (Gamal, 2009). The infection starts in bronchi leading to patchy area of consolidation in one or more lobes, which breakdown to form abscess, there is much destruction, empyema and pneumothorax may occur from rupture of pulmonary abscess into the pleural cavity (Roderick *et al.*,1994).

Since empyema, pneumothorax and pneumatoceles are so commonly seen with staphylococcal pneumonia they are considered part of natural course of the disease and not complication, complication of staphylococcal pneumonia include staphylococcal pericarditis, meningitis, osteomyelitis and multiple metastatic abscesses in soft tissues (Gamal, 2009). Futhermore, these serious infections have emerged more frequently among non-hospitalized patients and are associated with strains that produce the panton-valentine leukocidin toxin. Also worrisome is that

these serious infections are frequently mediated by methicillin-resistant *Staph. aureus* (Betty *et al.*, 2007b).

2.2.4 Clinical Feature

A localized *Staph. aureus* infection appears as a "pimples" hair follicle, or abscess, there is usually an intense, localize painful inflammatory reaction that undergoes central suppuration and heal quickly when the pus is drained. Secondary localization within organ or systems is accompanied by the symptoms and signs of organ dysfunction and intense focul suppuration (Brooks *et al.,* 2007).While pneumonia, in young, previously healthy adults with a preceding influenza-like illness characterized by severe respiratory symptoms, hemoptysis, high fever, leukopenia, very high C-reactive protein level (>400 g/L), hypotension, and a chest x-ray showing multilobular cavitating alveolar infiltrates (Rubinstein *et al.,* 2008). Toxic shock syndrome is manifested by an onset of high fever, vomiting, diarrhea, myalgias , a scarlotiniform rash, and hypotension with cardiac and renal failure in the most sever cases,it occurs within 5 days after onset of the menses in young women who use tampons. Food - poisoning of *Staph. aureus* Characterized by short incubation period (1-8 hours) and violent nausea, vomiting, and diarrhea and rapid convalescence without fever (Brooks *et al.*, 2007).

2.2.5 Epidemiology

Staph. aureus is colonizer of various skin and mucosal surface. Human infections because carrier state is common among human population , infections are frequently acquired when the colonizing strain gains entrance normally sterile site as a result of trauma or abrasion to the skin or mucosal surface, however the traumatic event that allows entry of the organism often may be so minor that it goes unnoticed, *Staph. aureus* also transmitted from person to person, upon transmission, the organisms may become established as part of the recipient‘s normal flora and later be introduce to sterile site by trauma or invasive procedures.

Alternatively, the organism may be directly introduced into normally sterile sites, such as by a surgeon or nurse during surgery, person-to-person spread of *Staph. aureus* Particularly those that have acquired antimicrobial resistance, most notably occurs in hospitals and presents substantial infection control problems, however, more recently serious *Staph. aureus* infections have been encountered in the community setting as well (Betty *et al.,* 2007a).

2.3 Laboratory Diagnosis

2.3.1 Specimens

Depending on site of infection it may be: Nasal swabs, urine, wound swab or blood transport by aims transport media (Cheesbrough , 2007).

2.3.2 Microscopy

Gram positive cocci in group of uni-forming size (about 1micro m), non-motile, non-capsulated (Cheesbrough , 2007).

2.3.3 Culture

Staph. aureus grow well aerobically, and in a carbon dioxide enriched atmosphere, Most strains grow well anaerobically but less well, temperature range for growth is 10-42 $^{\circ}$C with optimum 35-37°C (Cheesbrough , 2007). Colonies of *Staph. aureus* after overnight incubation at 37°C, on nutrient agar are smooth, low convex, glistening, densely, opaque, and of a butyrous consistency. Older colonies become translucent and sticky (Collee *et al.,* 2000). On blood agar and chocolate (heated blood) agar, *Staph .aureus* produces yellow to cream or occasionally white 1-2 in diameter colonies. Some strains are Beta-heamolytic when grow aerobically. Colonies are slightly raised and easily emulsified on a slide, on MacConkey agar; smaller (0.1-0.5 mm) colonies are produced after overnight incubation at 35-37°C. Most strains are non-lactose fermentation, Manitol salt agar is useful differential and selective medium for recovering *Staph. aureus* (Cheesbrough, 2007).

2.3.4 Biochemical Reactions

Staph. aureus which is catalase positive that differentiate it from Gram-positive cocci include *streptococci* .but special tests to differentiate *Staph. aureus* from other species in the same genus staphylococci include co-agulase, DNA-ase (Cheesbrough, 2007) the heat-stable nuclease(thermonuclease, TNase). *Staph. aureus* ferment three pattern of sugar include mannitol, trehalose and sucrose with acid production .and sensitive to 5micro g novobiocin disc. *Staph. aureus* produce acetoin (Voges-Proskauer),gelatinase and alkaline phosphatase positive. Variable strains produce urease and esterase and ferment lactose (Collee *et al.,* 2000).

2.3.5 Serology Tests

Antibiotic to teichoic acid, a major cell wall components of Gram-positive bacteria are usually produced in long-standing or deep-seated staphylococcal infections, such as osteomyelitis, this procedure, if required, is usually perform in reference laboratories (Betty *et al.,* 2007a). Several latex agglutination test kits are available to identify *Staph. aureus* based on detection of clumping factor , or protein A , a latex particles are sensitized with fibrinogen and immunoglobulin G (Cheesbrough, 2007).

2.4 Bacteriophage Typing

Strain of *Staph. aureus* can be differentiate into different phage type by observation of their pattern of susceptibility to lysis by a standard set of *Staph. aureus* bacteriophages, virulent phages can lysis of *Staph. aureus* thus produce a clearing in the lawn of growth. Phage types are designated according to the phage able to cause this effect. Many of MRSA strains are not typable with standard and additional or experimentals phages (Greenwood *et al.,* 2007). Most enterotoxin-producing strain of *Staph. aureus* belong to phage group III (Cheesbrough, 2007). Most *Staph. aureus*-exfoliative toxin producing strains belong to phage group II (Greenwood *et al.,* 2007).

2.5 Treatment

2.5.1 Penicillins

Bactericidal agents benzylpenicillin (i.m., i.v. administration) and penicillin V (oral) are used to treat infections caused by streptococci, pneumococci, clostridia and when sensitive, also staphylococcal infections, meningitis, gonorrhoea, syphilis and anthrax. Flucloxacillin and cloxacillin are used to treat *beta*-lactamase (penicillinase) producing staphylococci. Ampicillin and amoxycillin are broad-spectrum penicillins, active against Gram positive bacteria (including enterococci) *H. influenzae*, and many coliforms (Mackie and McCartney, 2000).

2.5.2 Carbenicillin and Ticarcillin

Are useful in treating infections caused by *P. aeruginosa*. Azlocillin and piperacillin are active against klebsiellae and are also anti-pseudomonal. Hypersensitivity reactions include anaphylaxis (IgE mediated), delayed hypersensitivity (IgG mediated), erythema nodosum, and skin rashes. Patients with penicillin hypersensitivity may also show allergy to cephalosporins. Anti-bacterial resistance to penicillins may occur due to *beta* lactamase production, cell membrane alterations reducing antibiotic uptake (Gram negative bacteria), or changes in penicillin-binding proteins as occurs with MRSA (methicillin resistant *Staph. aureus*). MRSA are usually resistant to many antibiotics (e.g. penicillins, tetracyclines, erythromycin, and sometimes gentamicin). Severe infections require treatment with vancomycin. MRSA vancomycin-intermediate strains have been reported (Susan *et al.*, 2006).

2.5.3 Co-trimoxazole

Is used to treat urinary and respiratory tract infections, *Pneumocystis pneumonia*, and invasive salmonellosis. Many enterobacteria are resistant. Side-effects include nausea and vomiting, rashes, mouth ulceration and occasionally thrombocytopenia and leucopenia. Side-effects are less with trimethoprim. Bacteristatic and

bactericidal agents Antibacterial agents are generally described as bacteristatic when, at usual dosages, they prevent the active multiplication of bacteria, e.g. chloramphenicol, tetracycline, and erythromycin (Cui *et al*., 2006).

2.5.4 Methicillin

Meticillin (INN, BAN) or methicillin (USAN) is a narrow-spectrum beta-lactam antibiotic of the penicillin class. It should not be confused with the antibiotic metacycline. It was previously used to treat infections caused by susceptible Gram-positive bacteria, in particular, beta-lactamase-producing organisms such as *Staph. aureus* that would otherwise be resistant to most penicillins, but is no longer clinically used(Susan *et al.,* 2006).

Its role in therapy has been largely replaced by flucloxacillin and dicloxacillin, however the term methicillin-resistant *Staph. aureus* (MRSA) continues to be used to describe *Staph. aureus* strains resistant to all penicillins. Methicillin is no longer manufactured because the more stable and similar penicillins such as oxacillin (used for clinical antimicrobial susceptibility testing), flucloxacillin, and dicloxacillin are used medically (Betty *et al.,* 2007b).

2.5.4.1 Mode of Action

Like other beta-lactam antibiotics, methicillin acts by inhibiting the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell wall of Gram-positive bacteria. It does this by binding to and competitively inhibiting the transpeptidase enzyme used by bacteria to cross-link the peptide (D-alanyl-alanine) used in peptidoglycan synthesis. Methicillin and other beta-lactam antibiotics are structural analogs of D-alanyl-alanine, and the transpeptidase enzymes that bind to them are sometimes called penicillin-binding proteins (PBPs) (Sajna *et al.,* 1999).

Methicillin is insensitive to beta-lactamase (also known as penicillinase) enzymes secreted by many penicillin-resistant bacteria. The presence of the ortho-

dimethoxyphenyl group directly attached to the side-chain carbonyl group of the penicillin nucleus facilitates the β-lactamase resistance, since those enzymes are relatively intolerant of side-chain steric hindrance. Thus, it is able to bind to penicillin-binding proteins (PBPs) and inhibit peptidoglycan crosslinking, but is not bound by or inactivated by β-lactamases (Susan *et al.*, 2006).

2.5.4.2 Clinical use

Methicillin is no longer used to treat patients. Compared to other beta-lactamase-resistant penicillins it was less active, could only be administered parenterally and had a higher frequency of interstitial nephritis, an otherwise rare side-effect of penicillins. But it serves a purpose in the laboratory to determine the antibiotic sensitivity of *Staph aureus* to other beta-lactamase-resistant penicillins (Tacconelli *et al.*, 2008).

2.5.4.3 Mechanism of Resistance

MRSA is capable of resisting β-lactamase resistant Antibiotics via the mecA gene. This is a gene that transcribes Penicillin-binding-protein 2A (PBP2A), resulting in the binding of these antibiotics, thereby preventing their function (Tacconelli *et al.*, 2008).

Resistance to methicillin is mediated via the mec operon, part of the *staphylococcal* cassette chromosome *mec* (SCC*mec*), resistance is conferred by the *mec*A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins, and carbapenems). This allows for resistance to all β-lactam antibiotics, and obviates their clinical use during MRSA infections. As such, the glycopeptide vancomycin is often deployed against MRSA (Hakim *et al.*, 2007).

2.5.4.4 Methicillin-Resistant *Staph. aureus* (MRSA)

Methicillin-resistant *Staph. aureus* (MRSA) was first recognized in Europe and the United States in the late 1960s (Benner and Kayser 1968; Barrett *et al.*, 1968) and

is now evidenced worldwide, MRSA is endemic in many hospitals throughout the world and particularly affects vulnerable patients, such as those who have undergone major surgery and patients in the intensive care unit .Although 50-60% of patients with MRSA are merely colonized example they carry the bacteria but do not have symptoms or an illness .Serious infections such as those involving the blood stream, respiratory tract and bones or joints do occur, these infections are then more difficult to treat than infections caused by methicillin-susceptible isolates and MRSA can easily among patients in hospital methicillin resistance is mediated through the *mec*A gene, which encodes a unique penicillin-binding protein. Community-acquired strains have been describe that can cause soft tissue infections, these strains often produce the panton-valentine leucocidin they can be distinguished from endemic hospital strains, from which it is believed that have arisen (Green wood *et al.,* 2007). In fact, MRSA accounts for a growing proportion of *Staph. aureus* isolated from hospitalized patients in many countries. According to 2003 National Nosocomial Infections Surveillance reports, the proportion of intensive care unit patients with *Staph. aureus* nosocomial infections resistant to oxacillin has increased from 30% in 1989 to 60% in 2003 (National Noscomial Infections Surveillance System Report ,2003). Between 2005 to early 2006 in Al-Zahra hospital, a state runs educational hospital in Isfahan, Iran, 67.2 % isolated rate of MRSA from patients who contracted nosocomial infection after hospital admission (Khorvash *et al.,* 2008).

Another study to detect MRSA from various clinical sample, conduct in the Department of Microbiology, J.N Medical College and Hospital AMU, Aligarh during the period from august 2005 to july 2007 found that (143) 33.25% isolate rate for MRSA and 7.97% of MRSA strains were resistant to vancomycin by himartshu method and 5.7% by the method of tenover and CO -Workers (Khan *et al* ., 2011). From 24 different hospital across 16 states in america between july

1998 to November 2001, 30 MRSA isolate from 30 patients, 23 patients were vancomycin treatment failure and 7 patients were treated successfully, once considered only a nosocomial pathogen, MRSA appeared in non-hospitalized patients in 1980, primarily among intravenous drug users (Khan *et al* ., 2011).

In 2003, Jernigan *et al* conducted a prevalence study of MRSA colonization among patients presenting to a university hospital by performing surveillance cultures at the time of hospital admission. Of the 974 patients cultured, 21% had *Staph. aureus* isolated, and 26 (2.7%) had MRSA, representing 12.7% of all patients colonized with *Staph. aureus* (Jernigan *et al.*, 2003). It may be possible that community-acquired MRSA is dependent on a geographic factor or specific high-risk population, such as, children in day care, inmates, sport teams, Native Americans, and other minorities (. Adcock *et al.*, 1998; Groom *et al.*, 2001; Lindenmayer *et al.*, 1998). Looked at routine screening for MRSA on admission to acute rehabilitation units and reported a 12% isolation rate for MRSA on newly admitted patients and 7% for in-house transfers (Manian *et al.*, 2002).

2.5.5 Vancomycin

2.5.5.1 Definition

Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria. It has traditionally been reserved as a drug of "last resort", used only after treatment with other antibiotics had failed, although the emergence of vancomycin-resistant organisms means that it is increasingly being displaced from this role by linezolid (Zyvox) available PO and IV and daptomycin (Cubicin) IV and quinupristin/dalfopristin (Synercid) IV, vancomycin never became the first-line treatment for *Staph. aureus* for several reasons: because of its poor oral bioavailability, it must be given intravenously for most infections, β-lactamase-resistant semi-synthetic penicillins such as methicillin (and its successors, nafcillin and cloxacillin) were subsequently developed, which

have better activity against non-MRSA Staphylococci and early trials used early impure forms of vancomycin ("Mississippi mud"), which were found to be toxic to the ears and to the kidneys these findings led to vancomycin's being relegated to the position of a drug of last resort(Asadullah *et al.,* 2003).

2.5.5.2 Pharmacology and Chemistry

Vancomycin is a branched tricyclic glycosylated nonribosomal peptide produced by the fermentation of the Actinobacteria species Amycolatopsis orientalis (formerly designated Nocardia orientalis). Vancomycin exhibits atropisomerism , it has multiple chemically distinct rotamers owing to the rotational restriction of some of the bonds. The form present in the drug is the thermodynamically more stable conformer and, therefore, has more potent activity (Kania *et al.,* 2004**).**

2.5.5.3 Mechanism of Action

Vancomycin acts by inhibiting proper cell wall synthesis in Gram-positive bacteria. Due to the different mechanisms by which Gram-negative bacteria produce their cell walls and the various factors related to entering the outer membrane of Gram-negative organisms, vancomycin is not active against Gram-negative bacteria (except some non-gonococcal species of *Neisseria*) (Kaur and Pathania , 2010).

To be specific, vancomycin prevents incorporation of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) peptide subunits into the peptidoglycan matrix; which forms the major structural component of Gram-positive cell walls.

The large hydrophilic molecule is able to form hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides. Under normal circumstances, this is a five-point interaction. This binding of vancomycin to the D-Ala-D-Ala prevents the incorporation of the NAM/NAG-peptide subunits into the peptidoglycan matrix (Brooks *et al.,* 2007).

2.5.5.4 Vancomycin- Resistant *Staph. aureus* (VRSA)

Vancomycin-resistant *Staph. aureus* refers to strains of *Staph. aureus* that have become resistant to the glycopeptide antibiotic vancomycin. With the increase of staphylococcal resistance to methicillin, vancomycin (or another glycopeptide antibiotic, teicoplanin) is often a treatment of choice in infections with methicillin-resistant *Staph. aureus* (MRSA) ,three classes of vancomycin-resistant *Staph. aureus* have emerged that differ in vancomycin susceptibilities: vancomycin-intermediate *Staph. aureus* (VISA), heterogenous vancomycin-intermediate *Staph. aureus* (hVISA), and high-level vancomycin-resistant *Staph. aureus* (VRSA) (Appelbaum, 2011).

High-level vancomycin resistance in *Staph. aureus* has been rarely reported. However, these strains may also be resistant to meropenem and imipenem, two other antibiotics that can be used in sensitive *Staphylococcus* strain (Appelbaum, 2011).

2.6 Prevention and Control

Staph. aureus are variably sensitive to many antimicrobial drugs, Resistant to penicillin depends on production of the enzyme penicillinase, a β-lactamase that open β-lactam ring (Greenwood *et al.*, 2007). The β-lactamase gene is encoded by plasmid, which transmitted by transduction, and conjugation, Penicillin-resistant strain of *Staph. aureus* are treated with flucloxacillin, methicillin and the cephalosporins independent of β-lactamase production. The *mec*A gene for nafcillin resistance resides on the chromosome, and the gene encodes a low-affinity penicillin binding protein (PBP2 or PBP2a) (Brooks *et al.*, 2007), MRSA strains resistant to all β-lactam agent, and often other agent such as aminoglycoside, and fluoroquinolones. Glycopeptides (vancomycin or teichoplanin) are the agents of choice in the treatment of systamic infection with MRSA (Greenwood *et al.*, 2007). Antibiotic treatment for MRSA depends on the

strain of MRSA as well as the invasiveness of disease, extremes of age, fever, cellulitis and lesion size, community-acquired MRSA (CA-MRSA) isolate are typically susceptible to trimethoprime, sulfamethoxazole (TMP-SMX), clindamycin, gentamicin while hospital-acquired MRSA (HA-MRSA) are usually resistant to these drugs, preferred antibiotic treatment for (CA-MRSA) infections include doxycycline, TMP-SMX and clindamycin are the most commonly used In the output patient setting doxycycline, TMP-SMX and clindamyin are commonly use (Johnson and Decker, 2008).

TMP-SMX has been shown in vitro to be bactericidal when compared to linezolid, rifampin, clindamycin, minocycline and moxifloxacin. TMP-SMX is appealing as drug of choice; it is relatively inexpensive and has good oral bioavailability (Johnson and Decker, 2008). Clindamycin is another good oral antibiotic for treating CA-MRSA skin and soft tissue infections; it inhibits ribosomal function and bacterial protein production, decreasing exotoxin production to minimize toxin-related complications (Micek, 2007). Clindamycin is not recommended for sever HA-MRSA infections due to high levels of resistance, with CA-MRSA infections clindamycin resistance varies regionally, so it use may be limited. Tetracycline, specifically longer acting minocycline and doxycycline are an othor option for treating MRSA infection, given their good oral bioaviability and tissue penetration (Micek, 2007). CA-MRSA is usually susceptible to tetracycline, but resistance has emerged slowly, tetracycline is often used concurrently with rifampin (Johnson and Decker, 2008). In the United States, *Staph. aureus* consider to by susceptible to vancomycin if the minimum inhibitory concentration(MIC) is equal or less to 2mg/ml, of intermediate susceptibility if the MIC between 4-8mg/ml, and resistant if the MIC is equal or more than 16mg/ml(Huang and Platt, 2003). Strain of *Staph. aureus* with intermediate susceptibility to vancomycin have been isolated in Japan, the united states, and several other countries, these are often

known as vancomycin intermediate *Staph. arueus*, or VISA, they generally have been isolated from patients with complex infection who have recieved prolonged vancomycin therapy, often there has been vancomycin failure. The mechanism of resistance is associated with increased cell wall synthesis and alterations in cell wall and is not due to *van*A gene found in *enterococci* (Hartman and Tomasz ,1984). *Staph. aureus* strains of intermediate are susceptibility to vancomycin usually are nafcillin resistant but generally are suscepitible to oxazolidinones and to quinopristin/ dalfopristin (Wootton *et al* .,2001). Since 2002, several isolates of vancomycin-resistant *Staph. aureus* (VRSA) strains were isolated from patients in the United States. The isolates contained vancomycin resistance gene *van*A from *enterococci* and nafcillin resistance gene *mec*A, both of the initial VRSA strains were suscepitible to other antibiotics, vancomycin resistance in *Staph. aureus* is major concern worldwide. Plasmid-mediated resistance to tetracyclines, erythromycins, aminoglycosides, and other drugs is frequent in staphylococci (Xxu *et al.*, 2010). Tolerance implies that *Staph. aureus* are inhibited by drug but not killed by it, there is great difference between minimal inhibitory and minimal lethal concentration of antimicrobial drug, patients with endocarditis caused by a tolerant *Staph. aureus* may have a prolong clinical course, compared with patients who have endocarditis caused by a fully susceptible *Staph. aureus*, tolerance may be attributed to lack of activation of autolytic enzymes in the cell wall (Brooks *et al.*, 2007).

There is no approved anti-*Staph. aureus* vaccines (Betty *et al.*, 2007b). The source of infection is shedding human lesions, fomites contaminated from such lesions and human respiratory tract and skin, although cleanliness, hygiene and aseptic management of lesions can control the spread of *Staph. aureus* from lesions, few method are available to prevent the wide dissemination of *Staph. aureus* from carrier like aerosols example glycols and ultraviolet irradiation of air but have little

effect, carriers can control by application of topical antiseptics to nasal or perneal, carriage side may diminish shedding of dangerous organisms. Rifampin coupled with a second oral anti *staphylococcal* drug sometimes provide long-term suppression and possibly cure of nasal carriage, this of therapy is usually reserved for major problem of *Staph. arueus* carriage because can rapidly develop resistant to rifampin, to diminish transmission within the hospital setting high risk patients, such as those in intensitive care units and patients transferred from chronic care facilities where prevalence is high, are frequently surveyed for anterior nares colonization. Patients who test positive by culture or PCR are placed upon contact precautions so as to minimize spread on the hands of the health care workers. Health care workers should strictly adhere to infection control policies by wearing gloves and wash hands before and after patients contact (Brooks *et al.*, 2007).

The control and prevention of MRSA involves early and reliable detection in the laboratory through surveillance, patients isolation when admitted to hospital, good professional practice by all health-care workers including compliance hand hygiene guidelines, effective hospital hygiene programs and the sensible use of antibiotics such measures have been very successful in Scandinavia and some other countries, but the prevalence of MRSA elsewhere, including the UK, Irland and Southern Europe, is much higher (Greenwood *et al.*, 2007).

Prevention is paramount for controlling HA-MRSA and CA- MRSA infections. Good hygiene and wound care should be reinforced with patients, family clinicians, for physicians and health care workers and hand washing with soap and water or with alcohol-based solutions is mandatory before and after patient contact. Contaminated fomites should be washed with detergent and hot water, person items should not be shared and contact sports with infected individuals should be avoided (Mylotte *et al.*, 2003). Medical personnel should also use contact

precautions (gloves, contact precaution) when examining the patient or draining a wound, nasal decolonization is controversial strategy but the CDC recommended the decolonization in patient with repeated MRSA in infection, despite other preventative measures (Growitz *et al.,* 2006). Nasal decolonization is achieved by applying antibiotic like Chlorhexidine, Mupirocin, TMP-SMX to the anterior nares twice daily for specific period of time (usually 5 days) (Boyce, 2001). Although usually effective in the short term, and recolonization is common. Outbreak control of CA-MRSA can be accomplished by identifying infection and applying therapeutic interventions early, improving hygiene and using antibiotics when necessary (Growitz *et al.,* 2006).

2.7 Genome Sequencing of *Staph. aureus*

Staph. aureus is a human pathogen that causes both nosocomial and community-acquired infections. The emergence of strains resistant to many antibiotics (methicillin-resistant *Staph. aureus* MRSA) and of highly virulent community-acquired MRSA that can cause fatal infections such as necrotizing pneumonia is of considerable concern even in countries with well-developed health surveillance systems (Naimi *et al.*, 2001; Torell *et al.*, 2005). In order to study mechanisms of staphylococcal antibiotic resistance and virulence, whole genome sequences of several different *Staph. aureus* strains have been determined. MRSA strains N315 and Mu50 were the first staphylococcal genomes to be sequenced (Kuroda *et al.,* 2001), which were followed by nine additional strains (Baba *et al.,* 2002; Diep *et al.,* 2006; Gillaspy *et al.*, 2006). All *staphylococcal* genomes are approximately 2.8 Mbp in size with a relatively low G+C content. Comparative analysis revealed that most regions of the staphylococcal genome are well conserved, whereas several large sequence blocks display high variability. *Staph. aureus* strains likely acquired these genomic islands horizontally and, at least initially, their integration into the genome must have required dedicated DNA recombination (integrase)

genes. Furthermore, variable blocks of genome sequence frequently carry virulence and antibiotic resistance determinants that aid in the development of Staphylococcal diseases. Variable regions can be classified as prophages, pathogenicity islands, or *Staphylococcal* cassette chromosomes. The overall combination of variable sequence elements and the encoded spectrum of virulence properties varies from strain to strain and appears to be reflective of the overall large spectrum of clinical disease manifestations in humans (Baba *et al.*, 2002; Baba *et al.*, 2003).

2.7.1 *arc* C Gene

*arc*C (Carbamate kinase) is one of the seven house-keeping genes produce by *Staph. aureus*, linear DNA (Amaral *et al.*, 2005).

Multi Locus Sequence Typing (MLST) is a well established typing method that looks at 7 house-keeping genes in *Staph.* These are genes that are always turned on (Edward *et al*., 2007).

Multilocus sequence typing (MLST) is a technique in molecular biology for the typing of multiple loci. The procedure characterizes isolates of microbial species using the DNA sequences of internal fragments of multiple housekeeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequence. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST) (Amaral *et al.*, 2005).

2.7.2 *mec* A Gene

*mec*A gene is a gene found in bacterial cells. The *mec*A gene allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. The most commonly known carrier of the *mec*A gene is the bacterium known as MRSA, found in *Staph. aureus* and *Strepto. pneumoniae* strains resistant

to penicillin-like antibiotics. In *Staphylococcus* species, *mec*A is spread on the SCC*mec* genetic element is part of a 21- to 60-kb Staphylococcal chromosome cassette *mec* (SCC*mec*) (Ubukata *et al.*, 1989).

The *mec*A gene does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is allowed to replicate as normal. The gene encodes the protein PBP2A (Penicillin binding protein 2A), PBP2A has a low affinity for beta-lactam antibiotics such as methicillin and penicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis (Deurenberg and Stobberingh, 2009).

The *mec* gene complex is composed of *mec*A, its regulatory genes, and associated insertion sequences. The class A *mec* gene complex (class A *mec*) is the prototype complex, which contains *mec*A, the complete *mec*R1 and *mec*I regulatory genes upstream of *mec*A, and the hyper-variable region (HVR) and insertion sequence IS*431* downstream of *mec*A. The class B *mec* gene complex (class B *mec*) is composed of *mec*A, a truncated *mec*R1 resulting from the insertion of IS*1272* upstream of *mec*A, and HVR and IS*431* downstream of *mec*A. The class C*mec* gene complex (class C *mec*) contains *mec*A and truncated *mecR1* by the insertion of IS*431* upstream of *mec*A, and HVR and IS *431* downstream of *mec*A. There are two distinct class C*mec* gene complexes; in the class C1 *mec* gene complex, the IS *431* upstream of *mec*A has the same orientation as the IS*431*downstream of *mec*A (next to HVR), while in the class C2 *mec*gene complex, the orientation of IS*431* upstream of *mec*A is reversed. C1 and C2 are regarded as different *mec* gene complexes since they have likely evolved independently. The class D *mec* gene complex (class D *mec*) is composed of *mec*A

and *Δmec*R1, but does not carry an insertion sequence downstream of *ΔmecR1* (Katayama *et al.,* 2001).

2.7.3 *van*A Gene

*van*A type vancomycin resistance operon genes, which can synthesize peptidoglycan with modified C-terminal D-Ala-D-Ala to D-alanine--D-lactate (Dutka-Malen, 1990)

van A has a Tn3-like transposon with a cluster of seven genes (*van*R, *van*S, *van*H, *van* A, *van*X, *van*Y and *van*Z)(Arthur and P. Courvalin, 1993), *van*A is ligase of broad substrate specificity (Bugg *et al* .,1991) responsible for production of dipeptidase which is incorporated in peptidoglycan precursor in place of D-alanyl – D alanine, *van*A is produced by *van*H dehydrogenease , these proteins are necessary for vancomycin resistance where the vancomycin binding site is altered (Arthur *et al.,* 1991).

2.7.4 *van* B Gene

*van*B ligase gene cluster is divided in to three sub types *van*B1, B2, B3 located on transposon Tn 5382 resulted from conjugation of plasmids (Carias *et al.,* 1998; Dahl *et al.,* 1999). Another glycopeptide resistance gene, *van*M reported from Chinaencodes D-Alanine: D-Lactase ligase and is related to *van*A, *van*B and *van*D gene and transferred by conjugation (Zhang *et al.,* 2010).

As there is increase in emergence and rapid dissemination of resistance to vancomycin that has become challenge to treat human diseases, it was felt necessary to screen presence of vancomycin resistance or vancomycin resistance like DNA sequences that are present in various organisms prior to carry out molecular characterization. Therefore, the presence and extent of *van* genes has been analysed with the sequence information available in biological databases and also by construction of phylogenetic tree. Possibly, this would provide important

clues before initiating any treatment with glycopeptide or other antibiotics and understand the nature of dissemination of antibiotics resistance in various organisms (Zhang *et al.*, 2010).

As in *van*A-type strains, acquired *van*B-type resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala (Arthur *et al.,* 1996). The organization and functionality of the *van*B cluster is similar to that of *vanA* but differs in its regulation, because vancomycin, but not teicoplanin, is an inducer of the *van*B cluster, the *van*B operon contains genes encoding a dehydrogenase, a ligase, and a dipeptidase, all of which have a high level of sequence identity (67%–76% identity) with the corresponding deduced proteins of the *van*A operon and the *van*R_BS_B regulatory genes that encode a 2-component system only distantly related to *van*RS (34% and 24% identity) (Evers and Courvalin, 1996). The function of the additional *van*W protein found only in the *vanB* cluster is unknown, and there is no gene related to *van*Z, on the basis of sequence differences, the *vanB* gene cluster can be divided into 3 subtypes: *van*B1, *van*B2, and *van*B3 (Dahl *et al.,* 1999). There is no correlation between the *vanB* subtype and the level of resistance to vancomycin (Patel *et al.,*1998).

CHAPTER THREE

3. Materials and Methods

3.1 Type of the Study

3.1.1 Study Approach

The study is a qualitative study, aimed to provide evidences about the existence of resistant strains belonging to the *Staph. aureus* among Shendi population and to enrich the genes library with Sudanese isolates .

3.1.2 Study Design

Cross sectional laboratory based study aimed to determine the frequency of MRSA and VRSA among patients with different clinical manifestations as well as to determine different mutations (if present) in all genes responsible for resistance phenomenon in the *Staph. aureus* which includes: *arc*C, *mec*A, *van*A and *van*B.

3.1.3 Study Area

Different hospitals and clinical centers located in Shendi City were included in the study as well as medical staff of these centers.

3.1.4 Study Population

Three hundred (n=300) patients suffering from different clinical manifestations were included in this study. The study was done using different clinical specimens these include: urine, nasal swabs, wound swaps, ear swaps and skin swaps and swaps from health carriers, during the period from October 2012 to September 2013.

3.1.5 Ethical Clearance

Proposal of this study was scientifically reviewed and passed by the Ethical Committee, Shendi University and submitted to the Federal Ministry of Health and form of consent was taken from all candidates participating in the study.

3.1.6 Data Collection

Data were collected by using a standard data questionnaire eliciting basic information on age, gender, symptoms and signs of disease suspected by *Staph. aureus* (UTI, wound infection, nasal swab, ear swab and skin swab). Additional information captured included patient demographics, social status, history of previous infections, and treatment (Appendix I).

3.2 Diagnostic Methods

3.2.1 Samples Collection

300 samples were collected from patient and clinical staff, for urine samples, five ml mid stream urine were collected in wide mouth screw–capped and leak-proof sterile containers which contain 0.1g/10 ml boric acid as a preservative. All swaps samples were collected by using sterile cotton swab wetted with sterile normal saline. All steps were conducted under aseptic conditions.

3.2.2Media and Culture Conditions

All clinical samples except urine were first inoculated in to nutrient agar(N.A) (Hi-Media, India) and MacConkey agar (Oxoid) plates whereas the urine samples were inoculated only on CLED agar (Hi-Media, India) plates. The plates were incubated at 37°C for 24–48 h, then Gram stain ,biochemical tests (catalase test , coagulase test ,culture in DNA media) finally sensitivity test were conducted in Muller-Hinton agar(M.H) (Appendix V).

3.2.2.1 Growth on Nutrient Agar

All Staphylococcal isolates were inoculated onto N.A (Hi-Media, India) and plates were incubated at 37°C for 24–48 h. The growth was observed and recorded (Appendix Π).

3.2.2.2 Growth on MacConkey Agar

All staphylococcal isolates were again inoculated onto MacConkey agar (Oxoid Media) and plates were incubated at 37°C for 24–48 h, the fermentation of lactose was observed and recorded(Appendix Π).

3.2.2.3Growth on Manitol Salt Agar

All staphylococcal isolates were again inoculated onto Manitol salt agar (Hi-Media, India) and plates were incubated at 37°C for 24–48 h. Manitol fermentation was observed and recorded (Appendix Π).

3.2.3Gram's Stain

Fixed and dried smears were prepared from the growth. Gram's stain was performed for each slide according to Cheesbrouch. (2006) and examined microscopically by oil immersion lens (X 100) (Appendix III ; Appendix V)**.**

3.2.4 Biochemical Tests

These tests include catalase test, coagulase test, DNAse test and novobiocin disk for sensitivity these tests were done according to (Cheesbrouch, 1989).

3.2.4.1Catalase Test

In a test tube containing 1ml of 3% H_2O_2, enough portion of colony of tested organism was picked by wooden stick and inserted inside the tube then watched for the appearance of air bubbles indicative of catalase activity (Mackie and McCartney, 2009) (Appendix III).

3.2.4.2Coagulase Test

Slide coagulase tests of all growing isolates were performed by emulsifying few pure colonies of staphylococci from nutrient agar on undiluted plasma. Tube coagulase tests were performed by diluting the plasma in freshly prepared normal saline (1:6). Three to four pure colonies were emulsified in 1 ml of diluted plasma and the tubes were incubated at 37°C. Readings were taken at 1 h, 2 h, 3 h, and 4 h and further incubated overnight at room temperature if no clot formation was

observed. *Staph. aureus* ATCC 29213 was used as control strain (Mackie and McCartney, 2009),(Appendix III).

3.2.4.3DNAse Test

DNAase test was done to *Staph. aureus* which produce DNAase enzyme as in using sterile loop, the test and control organisms were inoculated on plate with medium containing DNA and incubated over-night at 37°C. The surface of the plate was covered by 1mol HCl solution. Clear area around the organism within 5 minutes from the addition of the acid was an indication of DNAse positive (McCartney *et al.*, 2002) (Appendix III).

3.2.5.4 Novobiocin Disk

Staph. aureus is susceptible to novobiocin antibiotics. The test were conducted according to McCartney *et al*., (2002) by inoculated the target microorganism in Mueller-Hinton agar (Hi-Media, India). Inhibition zones were measured after 24h and as recommended by the National Committee for Clinical Labrotary Standards (NCCL, 2000) (appendix III).

3.3 Determination of Resistant Isolates

3.3.1 Conventional Methods

3.3.1.1 Detection of MRSA

MIC of Methicillin (Hi-Media, India) and Vancomycin (Lilly Pharma, Giessen, Germany) were determined by agar dilution method. Briefly, gradient plates of Mueller-Hinton agar (Hi-Media, India). By direct colony suspension method 0.5 McFarland equivalent inoculum were prepared in normal saline from 18–24 h agar plate culture. All strains were spotted onto gradient plates. Plates were incubated overnight at 35°C for any visible resistant.

3.3.1.2 Detection of VRSA

Disc diffusion test of vancomycin (30 μg), mehecillin (5 μg) was carried out using Kirby-Bauer Method. Mueller-Hinton agar plates were overlaid with the inoculum

(turbidity equivalent to that of a 0.5 McFarland Standard) of the *Staph. aureus* clinical strains. Zone diameters were measured at 24 and 48 h according to guidelines recommended by Clinical and Laboratory Standards Institute (CLSI) (Wayne., 2007). *Staph. aureus* ATCC 29213 was used as reference strain (Appendix III).

3.3.1.3 Determination of MIC

Minimal inhibitory concentration (MIC) of methicillin was determined by tube dilution method. Briefly, gradient tubes of nutrient broth (Hi-media) were prepared with *S. aureus* and serial dilution of methicillin (0.25-256 µg/ml), the serial dilution in tubes incubated overnight at 35°C for 24 h for assessing the visible growth.

3.3.2 Molecular Methods

3.3.2.1 DNA Extraction by Phenol Chloroform Method

According to (Jain *et al*., 2002) 200µl of decontaminated sample was placed in boiling water bath at 100°C for 10 min. It was followed by incubation at 56°C for 3 hours after addition of equal amount of lysis buffer (Tris 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 10µl of Proteinase K (10mg/ml). The sample was then vortexed and boiled at 100°C for 10 minutes to inactivate proteinase K. DNA purification was done by addition of equal volume of Phenol: Chloroform (24:1) followed by chloroform only. The aqueous phase was finally transferred in 2.5 volume of chilled ethanol and sodium acetate (0.3M final concentration.) was added. Tubes were kept at -20°C overnight. The sample was centrifuged at 10,000 rpm for 10 minutes and the plasmid DNA pellet was washed with 70% chilled ethanol by centrifugation. The pellet was allowed to air dry and finally suspended into 25µl of D.W. (sterile) for PCR analysis (Appendix IV).

3.3.2.2 Sets of Primers used to Detect Resistant Genes

One set of primer were used to determine MRSA through *mec*A gene while two sets of primers were used to check the occurrence of VRSA these are *van*A and *van*B genes. More confirmation was adopted foe all *Staphylococcus* isolates by using one set of primer to amplify *arc*C gene.

3.3.2.2.1 Detection of *arc*C gene

Carbamate kinase gene (*arc*C), one of the housekeeping genes used for multilocus sequence typing (MLST) of *Staph. aureus*, was selected for this study(Table 1)

***arc* –Up** 5'TTGATTCACCAGCGCGTATTGTC3'

***arc*-Dn** 5' AGGTATCTGCTTCAATCAGCG3'

3.3.2.2.2 Detection of *mecA* gene

The oligonucleotide primers for *mecA* gene

(*mec*A F 5' GTAGAAATGACTGAACGTCCGATGA 3' and

mecA R 5' CCAATTCCACATTGTTTCGGTCTAA 3')

3.3.2.2.3 Detection of *vanA* and *vanB* genes

Oligonucleotide primers for *vanA*

(*vanA* F 5'CATGAATAGAATAAAAGTTGCAATA 3'and

vanA R 5'CCCCTTTAACGCTAATACGACGATCAA 3') and *vanB*

(*vanB* F 5' GTGACAAACCGGAGGCGAGGA 3' and

*vanB R*5'CCGCCATCCTCCTGCAAAAAA 3'.

3.3.2.3 Preparation of Master Mix

Before starting master mix preparation, hood was disinfected using 70% ethanol before and after preparation of each batch, then sterilized further by turning on the UV light for at least 20 min.

22 µl of master mix was prepared for one reaction using kit (VIVANTIS Co., Ltd., Selangor Darul Ehsan, Malaysia) as follow:

2.5 µl of 10x buffer was placed in sterile eppendorf tube (1x), 0.3 µl from 10mM forward primer was added (0.12mM), 0.1 µl from each dNTP 50 mM (0.2 mM), 1.5 µl of 25mM $MgCl_2$ (1.5mM), 0.125 µl of 500 units at 5U/µl Taq polymerase (2.5 units), 0.3 µl from 10mM reverse primer (0.12mM), the volume was completed to 22 µl by adding 16.875 µl of sterile distilled water, the contents of master mix was vortexed after addition of each item and lastly 3µl of template DNA was added.

In negative control 3µl of sterile distilled water was added, while DNA extracted was used as positive control (Appendix IV).

3.3.2.4 PCR Amplification

The reaction mixtures were then put in the thermal cycler (CONVERGYS® ltd Peltier Thermal Cycle) that carried out the following PCR program: initial 5 minutes denaturation step at 94°C for one cycle followed by repeating cycles of denaturation (30 seconds at 94°C), annealing (45 seconds at 58°C) and extension (40 seconds at 72°C) for 35 cycles, followed by a 5 minutes final extension step at 72°C.

3.3.2.5 Preparation of Agarose Gel

500 ml of 1X Tris/borate/EDTA (TBE) (Appendix X) was prepared to prepare the gel and to fill the electrophoresis tank, 1.5 gram of agarose powder (AppliChem) was added to 100 ml of electrophoresis buffer (TBE) in an Erlenmeyer flask for preparation of 1.5% agarose, the agarose solution was heated in hot plate to allow all of the grains of agarose to dissolve after covering the flask with aluminum foil to prevent evaporation, then the solution was cooled down to 60^{o}C and 5 µl of 0.5 um/ml ethidium bromide (Et Br) (Appendix XI) was added, 1.0 mm comb positioned above the gel casting tray before pouring the liquid agarose gel to permit complete well formation when the agarose solidify, after solidification comb was gently removed and enough electrophoresis buffer was added to the tank

to cover the gel (about 1 mm of depth), the top of the wells were submerged(Appendix IV).

3.3.2.6 Loading of Samples and Electrophoresis

8 µl of PCR product from each sample were mixed with 2 µl of loading dye and then the mixtures were delivered into the well. 7 µl of DNA ladder (marker) length 100 bp ladder with fragments ranging from 100 bp to 1000 bp were added to one well in each run to estimate the size of tested DNA sequence. The gel electrophoresis apparatus was connected to a power pack (Serva BluePower 500, Germany). The electrophoresis was performed at 50 V for 30 minutes (Appendix IV).

3.3.2.7 Visualization of PCR Product

After electrophoresis period the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Target DNA fragments specific for *mec*A, *arc*C, *van*A and *van*B gene complex were viewed under ultraviolet transilluminator (SYNGENE, UK). Lastly the gel was transferred to gel documentation system for photography documentation (Appendix IV).

3.4 Sequencing of Target Genes and Detection of Mutations

The target genes are *arc*C basic genes produce by *Staph. aureus* detect to confirm the *Staph. aureus* then, *mec*A genes ,*van*A and *van*B genes and the sequencing done in South Korea after transport by DHL company for all positive bands to Macrogens Inc(info@macrogen.com) and the mutation was detected after compared with the sequencing in NCBI (Nucleotides) by using blast/microbes sequencing.

Table 1. Sequences of Primers used in the Study

Primer specifity	Primers	Primer pair Sequence (5' ---3')	Product size (bp)	Ann. temp	Reference
Carbamate kinase (arcC)	arc F arc R	5'TTGATTCACCAGCGCGTATTGTC3' 5' AGGTATCTGCTTCAATCAGCG3'	56	55 C^0	(Enright *et al*., 2000)
mecA	mecA F mecA R	5'GTAGAAATGACTGAACGTCCGATGA3' 5'CCAATTCCACATTGTTTCGGTCTAA3'	310	55 C^0	(Hare and Malay, 2006)
van A	vanA F vanA R	5'CATGAATAGAATAAAAGTTGCAATA3' 5'CCCCTTTAACGCTAATACGACGATCAA3	1030	64 C^0	(Hare and Malay, 2006)
van B	van B F vanB R	5'GTGACAAACCGGAGGCGAGGA 3' 5'CCGCCATCCTCCTGCAAAAAA 3'	433	59 C^0	(Hare and Malay, 2006)

CHAPTER FOUR

4. Results

4.1 Epidemiological Findings

This is a cross-sectional laboratory based study aimed to determine the prevalence of methicillin and vancomycin resistance *Staph.aureus* among patients with different disease as well as nasal swaps from medical health carriers from different hospitals and Shendi University, in which 300 clinical samples (n=300) were collected.

4.1.1 Gender

The results obtained in this project explained clearly that both sexes were infected with *Staph. aureus* with different ratio; 195 (65%) were females and 105 (35%) were males giving an average sex ratio of 1.9:1.0 as shown in Figure 1.

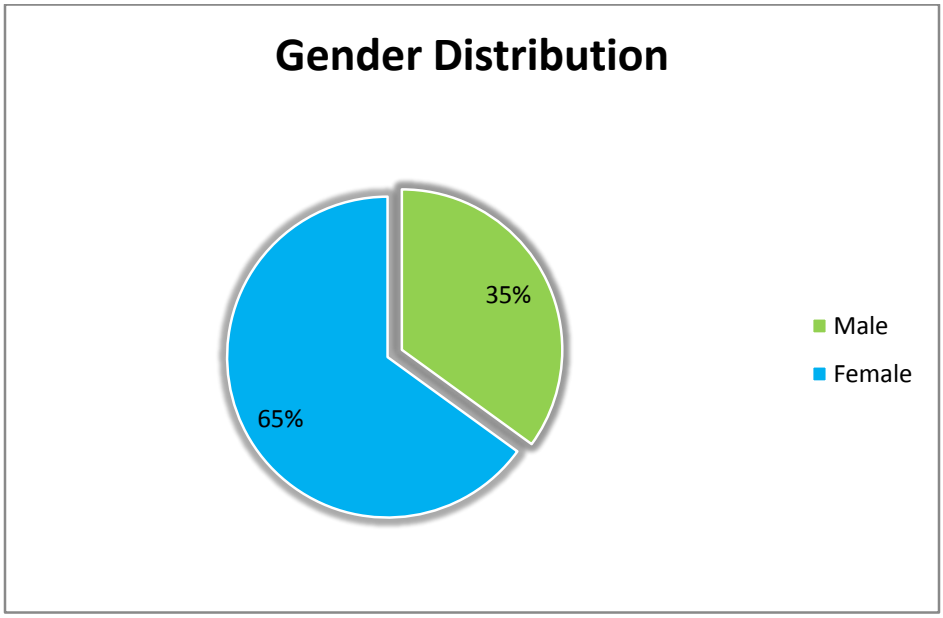


Figure 1. Distribution of samples in the study according to the gender

4.1.2 Age Group

All enrolled patients were classified into four age groups; age group one: less than 20 years old with lowest frequency 15(7.5%), age group two (20-39 years old) with the highest frequency 71(35.5%), age group three (40-59 years) with moderate to high frequency 66(33 %) and age group four more than 60 years with frequency 48(24%), *S.aureus* was isolated from all age groups as shown in Figure 2.

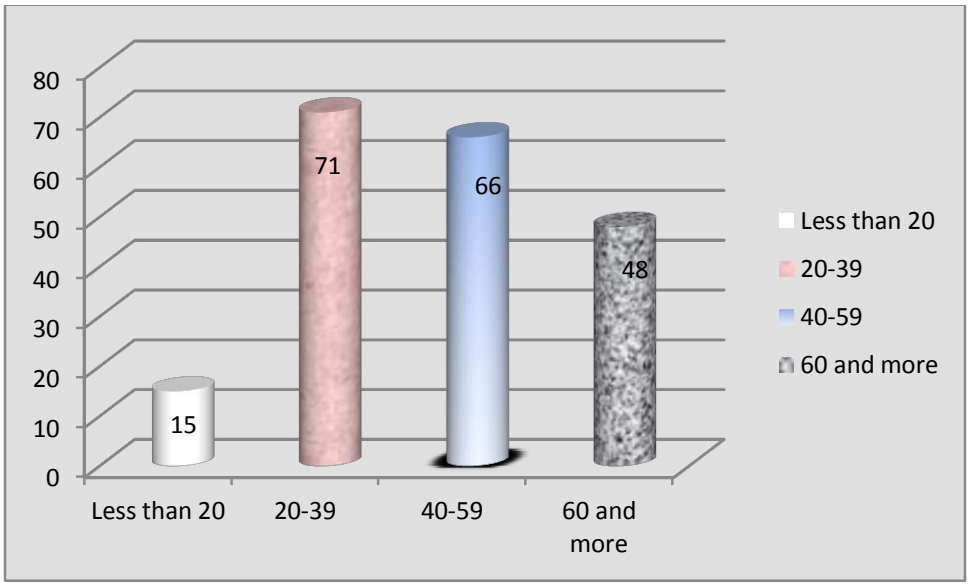


Figure 2. Distribution of age groups among enrolled patients.

4.1.3 Distribution of *S. aureus* in Different Clinical Specimens

The frequency of *S. aureus* isolated from different sources is shown in Figure 3. 80 (40%) isolates were from urine, 60(30%) were from wound, 50(25%) were from nasal and 10(5%) were f rom ear (Figure 3).

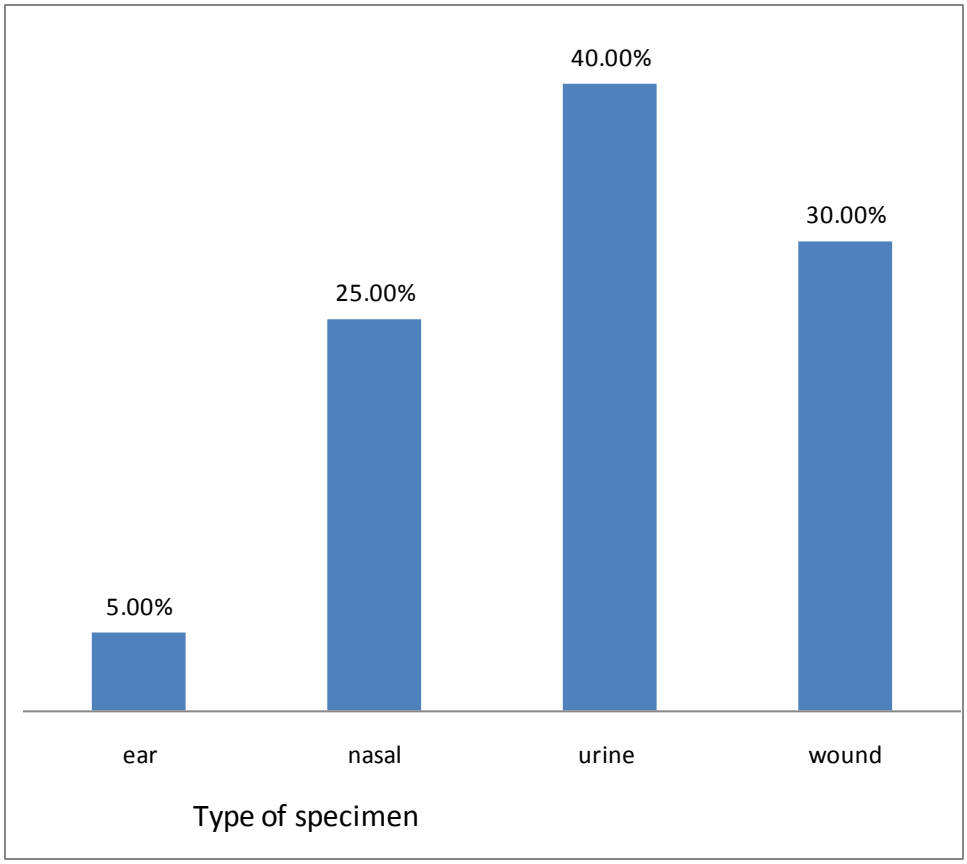


Figure 3. Frequency of *S. aureus* among different clinical samples

4.2. Bacteriological Findings

4.2.1 Phenotypic Properties

Gram's stain, colony morphology and different biochemical reactions including catalse test, coagulase test, DNase test, culture in Manitol salt agar and sensitivity to novobiocin disk were used for initial identification. The results of these tests were listed in color plates 1 to 7 (Appendix V**)**

4.2.2 Frequency of the Isolates

The data obtained in this study confirmed clearly the existence of *Staph. aureus* in 200 (67 %) cases from the 300 total samples. On the other hand species other than *Staph.aureus* were detected in this study, as follows: Gram negative rods 40 (13%), Gram positive cocci 260(86.7%). As shown in Table 2 Gram's result.

Table(2). Results of Gram's stain of the total 300 samples

	Frequency	Percentage %
Gram positive	260	86.7
Gram negative	40	13.3
Total	300	100

4.2.3 Biochemical test

4.2.3.1 Catalase test

Catalase test for 260 samples negative result (*Strepto cocci*) 10(3.8%), *Staphylococcus* (catalase positive) 250 (96.2%).

Table (3) shows the catalase test result to the cocci bacteria

Test	Frequency	Percentage %
Catalase positive	250	96.2
Catalase negative	10	3.8
Total	260	100

4.2.3.2 Coagulase test

Table (4) shows coagulase +ve and –ve result

Test	Frequency	Percentage %
Coagulase positive	200	80
Coagulase negative	50	20
Total	250	100

4.3 Antibiogram

4.3.1 Frequency of MRSA and VRSA

The results of Kirby-Bauuer disk diffusion method indicated that MRSA were isolated from 123/200 (61.5 %), while VRSA were also detected with frequency 8/200 (4%), (Fig. 4, Table 5).

Table (5) Frequency of MRSA among Enrolled Patients

Test	Frequency	Percentage %
MRSA	123	61.5
MSSA	77	38.5
Total	200	100

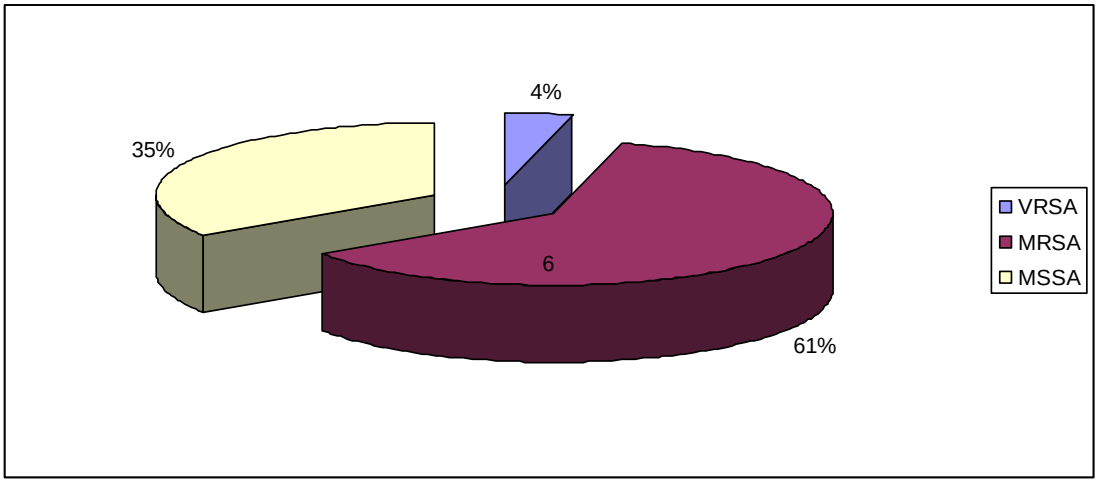


Figure 4. Percentage of VRSA versus MRSA among Study Subjects

4.3.2 Minimum Inhibitory Concentration (MIC) for MRSA

A total of 10% of isolates were found to be resistant to Methicillin(<0.25 and >2µg/ml), about 70% were inhibited by 4µg/ml, 10% by 8 µg/ml, 4% by 16µg/ml, 2% by 32µg/ml & 2% by 64µg/ml, 1%by 128µg/ml and 256µg/ml, as show in the table

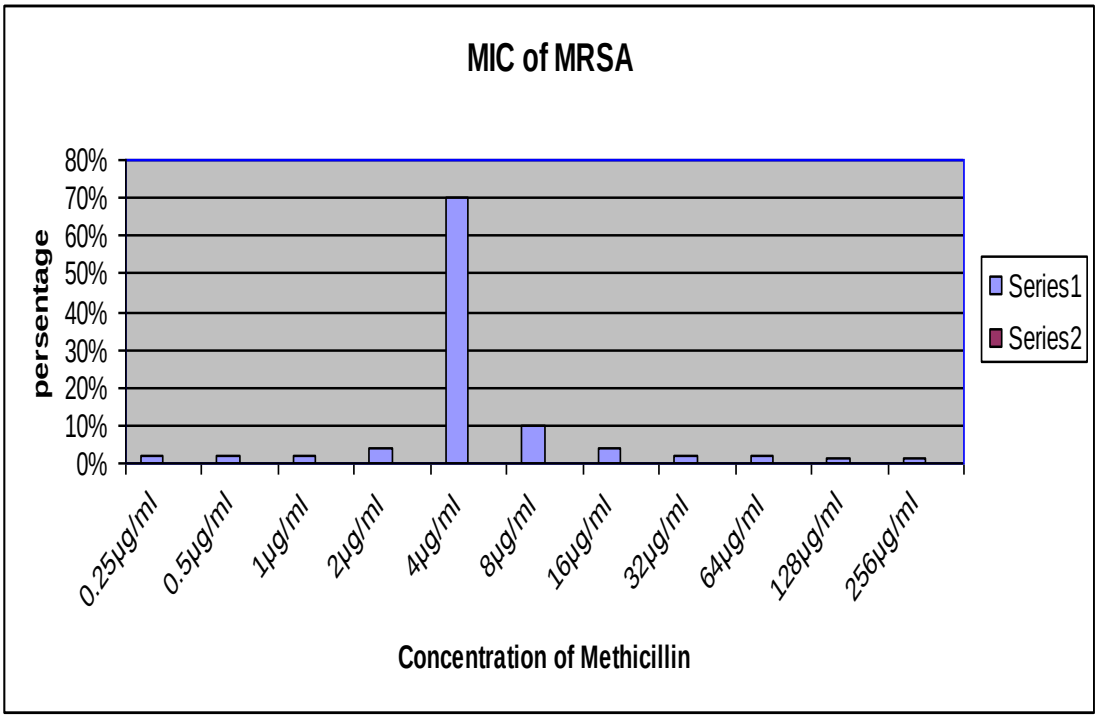


Figure 5. **Minimum Inhibitory Concentration (MIC) Range in Different MRSA Isolates**

4.3.3 The Prevalence of MRSA in the Different Clinical Samples

The frequency of MRSA isolates were 37.5, 29, 23 and 10.5 % in urine, wound, nasal and ear cultures, respectively (Figure 6)

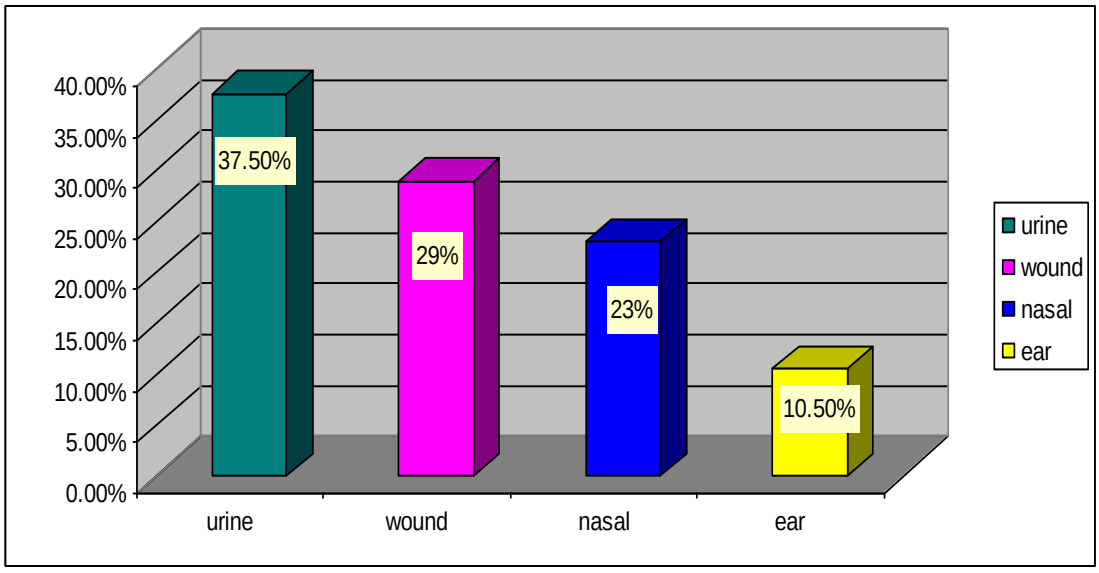


Figure 6 Frequency of MRSA isolates among different clinical samples

4.4. Molecular Findings

4.4.1 Extraction of DNA

Three protocols were used for extraction of the DNA from target species. Pure DNA was obtained when using phenol chloroform method as illustrated in Figure7

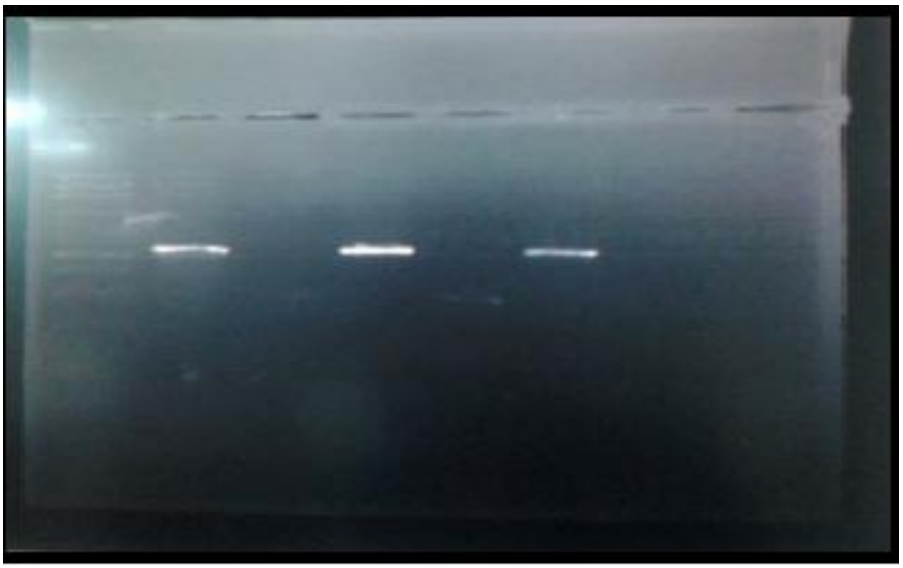


Figure 7. Pure DNA of *Staph.aureus* on on 1% agarose gel after using phenol chloroform method

4.4.2 Detection of *arcC* Gene by Polymerase Chain Reaction

All 123 methicillin-resistant *S. aureus* (MRSA) and 77 methicillin-sensitive S. *aureus* (MSSA) strains were tested positive for *arc*C genes as illustrated in Figure8.

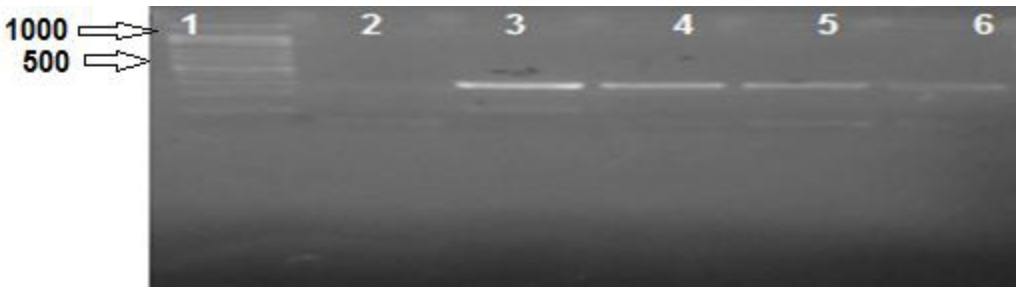


Figure 8. 2% agarose gel electrophoresis of PCR products. Lane 1: 100 bp molecular weight marker, Lanes 2: negative for *arc*C gene , Lanes: 3,4,5,6 are specimens under test showing positive results for *arc*C as indicated by 456 bp PCR ampilicon.

4.4.3 Detection of *mecA* Gene by Polymerase Chain Reaction

All the methicillin-resistant *Staphylococci* (MRSA) which included (123), were tested for the presence of *mec*A, 72/ 123 (58.5%) were positive for *mec*A gene and 51/ 123 (41.5%) were negative for *mec*A gene where as the MSSA isolates were negative for the *mec*A gene (Figure 9)

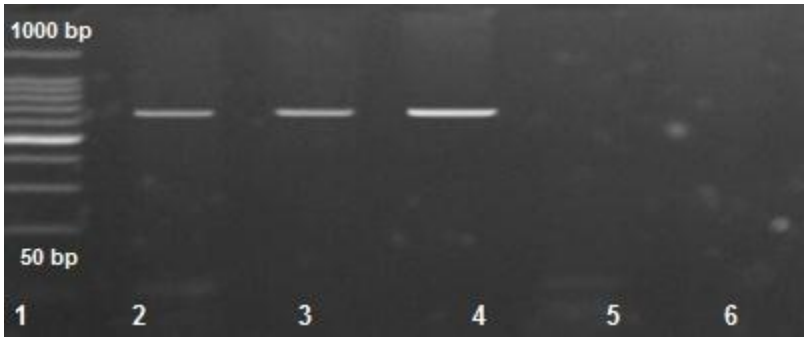


Figure 9. 2% agarose gel electrophoresis of PCR products. Lane 1: 50 bp molecular weight marker, Lanes 2,3,4 are positive for *mec*A as indicated by 310 bp PCR ampilicone, Lanes 5 and 6 are negative for *mec*A, (methicillin susceptible *S. aureus*)

4.4.4 Detection of *vanA* and *vanB* genes

van A gene was not detected in any of the tested strains while *van*B was detected only in 3/8 (38%) of the isolates, as was indicated by a band of 433 bp. (Figure 10).

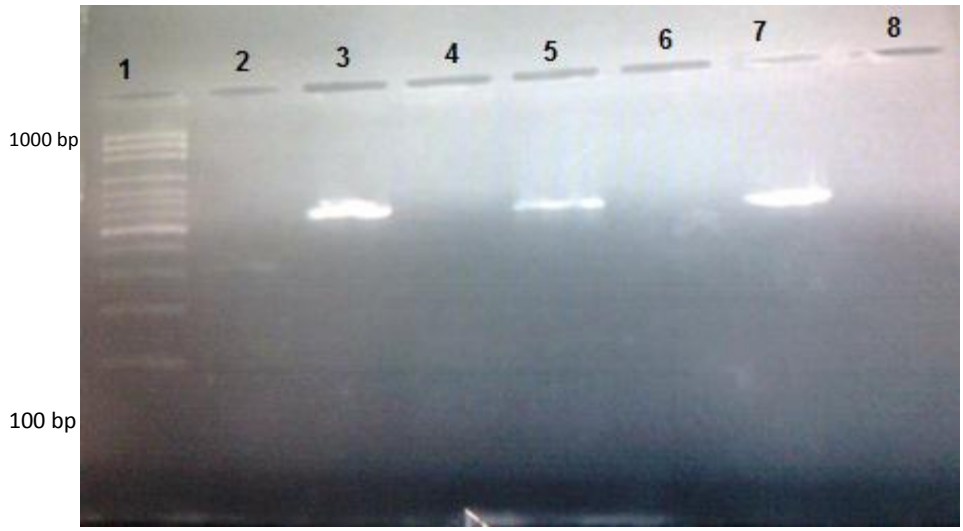


Figure 10. 2% agarose gel electrophoresis of PCR products. Lane 1: 50 bp molecular weight marker, Lanes 3,5and 7 are positive for *vanB* as indicated by 433 bp PCR ampilicone, Lanes 2,4,6 and 8 are negative for *vanB*, (vancomycin susceptible *S. aureus*)

Table (6). Distribution of Different Genes among MRSA Isolate

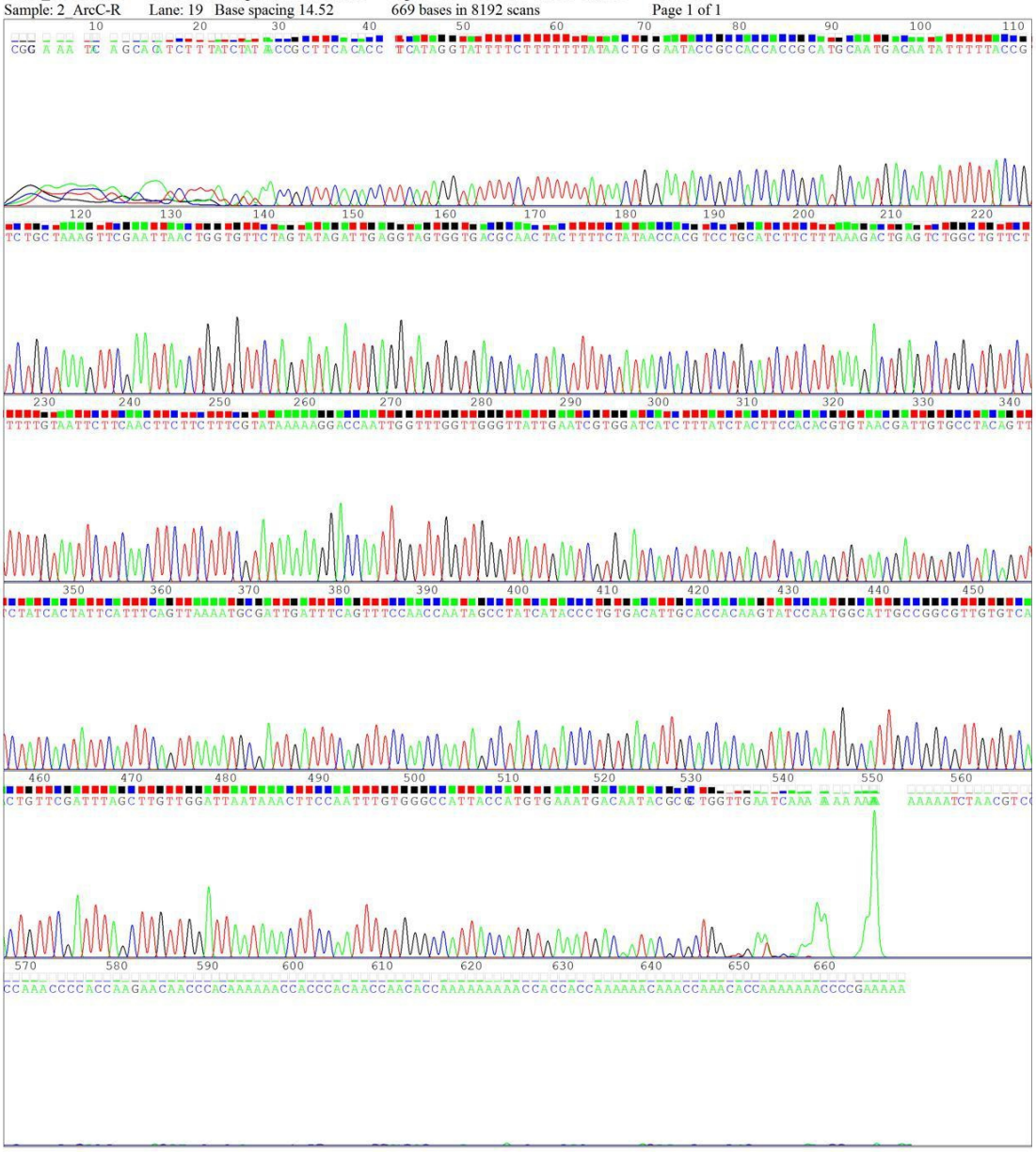
Genes name	Positive	Percentage %	Total
***arc*C gene**	150	100	150
***mec*A gene**	72	48	150
***van*A gene**	0	0	8
***van* B gene**	3	38	8

4.5 Genes Sequencing and Detection of Mutations

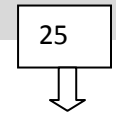
*arc*C and *mec*A sequence and sequence analysis was performed (Macrogene, Korea) and the sequence alignment was carried out. The analysis of the *mec*A sequence and *arc*C revealed that there is no obvious mutation in both genes (Figure 11.12). BLAST search at the GenBank database with the *mec*A sequences for other species of *S. aureus* displayed that were clearly closely related to SA268 sequ Sequence ID: gi|670938612|gb|CP006630.1| and NW19A Sequence ID: gi|752851829|gb|KM369884.1|, respectively with a nucleotide sequence identity of 100%.

A)

File: 2_ArcC-R.ab1 Run Ended: Aug 28, 2014, 1:48:21 Signal G:3568 A:3877 T:6329 C:4616 Comment:
Sample: 2_ArcC-R Lane: 19 Base spacing 14.52 669 bases in 8192 scans Page 1 of 1



B)



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AAAAGGACCAATTGGTTTAGTTGGGTTATCAAATCGTGGATCATCTTTATCTACTTCCACACGTGTTACGATTGTGC
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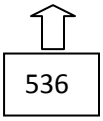

C)

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FCIKRTNWFSWVIKSWIIFIYFHTCYDCAYSSITIHFS*NAIDFSFQPIAYHTL*HCTTSIQWHCRRCVTVRFSLLD**TSNL
WAITM*NDGTRWWVRFL

D)

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Sbjct  2735571  ACCGCATGCAATGACAATATTTTTACCGTCTGCTAAAGTTCGAATTAACTGGTGTTCTAG  2735630

Query  145      TATAAATAGAGGTAGTGGTGACGCAACTACTTTTCTATAACCACGTCCTGCATCTTCTTT  204
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Sbjct  2735631  TATAGATTGAGGTAGTGGTGACGCAACTACTTTTCTATAACCACGTCCTGCATCTTCTTT  2735690

Query  205      AAATACTGAGTCTGGCTGTTCTTTTTGTAATTTTTCAACTTCTTCTTTTGTATAAAAAGG  264
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Query  445      CGGCGTTGTGTCACTGTTCGATTTAGCTTGTTGGATTAATAAACTTCCAATTTGTGGGCC  504
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Sbjct  2735991  ATTACCATGTGAAATGACAATACGCGCTGGTG  2736022
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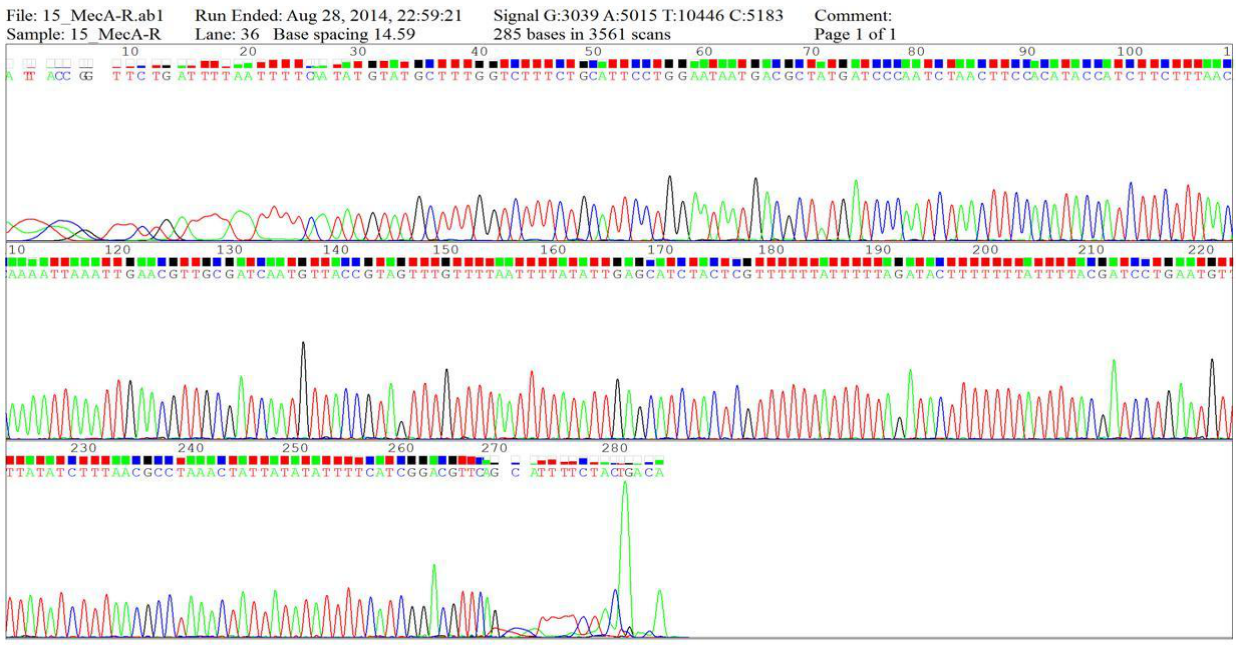
Figure 11. A: Electrophotogram of *arc*C gene, B: Nucleotide sequence of *arc*C gene, C: alignment Amino acid sequence alignment of *arc*C-gene, D: result of alignment of *arc*C ampilicon with *Staphylococcus aureus* subsp. *aureus* SA268 sequ Sequence ID: gi|670938612|gb|CP006630.1|

A)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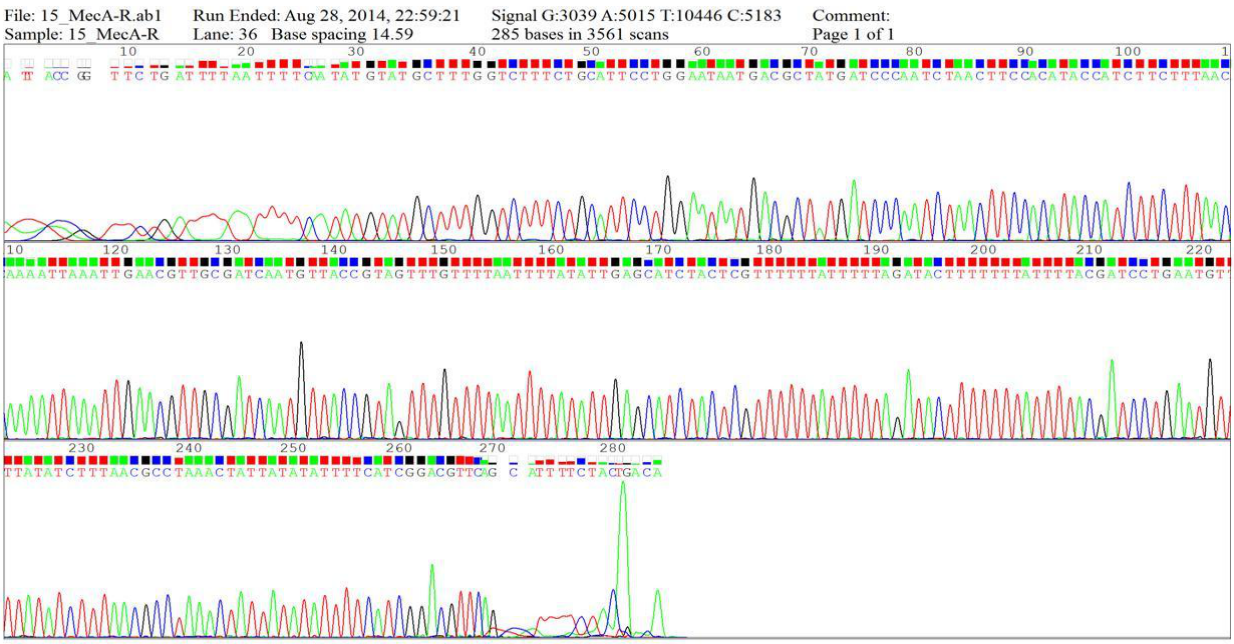
Sample: 15_MecA-R Lane: 36 Base spacing 14.59 285 bases in 3561 scans Page 1 of 1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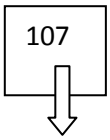
Sample: 15_MecA-R Lane: 36 Base spacing 14.59 285 bases in 3561 scans Page 1 of 1

B)



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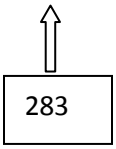

C)

YPVA*RVGI*TFRIVK*KKYLKIKNE*MLNIKLKQTTVTLIATFNLILLKKMVCGS*IGIIASLFQECRKTKAYILKI*NQNVVK
F*TETMVELIQ*LDALGYKHSGS*NKKSI*K*KTSRCSI*N*NKLR*H*SQRSI*FC*RRWYVEVRLGS

D)

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15496

Query    167       GTTAGATTGGGATCATAGCGTCATTATTCCAGGAATGCAGAAAGACCAAAGCATACATAT
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Figure 12. A: Electrophotogram of *mec*A gene, B :Nucleotide sequence of *mec*A gene, C: alignment Amino acid sequence alignment of *arcC*-gene, D: result of alignment of *mec*A ampilicon with *Staphylococcus aureus* subsp. NW19A chromosome mec type V 5C2&5c Sequence ID: gi|752851829|gb|KM369884.1|

CHAPTER FIVE

5. DISCUSSION

Staphylococcus aureus is one of the most common causes of nosocomial infections, especially pneumonia, surgical site infections and blood stream infections and continues to be a major cause of community-acquired infections. Methicillin-resistant *S. aureus* (MRSA) was first detected approximately 40 years ago and is still among the top three clinically important pathogens (Van Belkum *et al*., 2001; Deresinski., 2005). The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to the semisynthetic penicillins (methicillin, oxacillin, and nafcillin), macrolides, tetracycline, and aminoglycosides has made the therapy of Staphylococcal disease a global challenge (Maranan *et al.,* 1997).

Not only has *S. aureus* developed resistance to methicillin, it has developed resistance to newer antibiotics. As new antibiotics are developed, it is likely that this highly-adaptable bacterium will develop resistance to them as well. Research in the field of genomics may aid in the treatment of MRSA infection. As researchers gain further understanding of the MRSA genome, the outlook for patients with this disease may improve.

This study investigated the antibacterial-resistance patterns of *S. aureus* isolated from clinical samples in different hospitals in Shendi City, Sudan. Results of the current study revealed that the prevalence of MRSA isolates is (61.5%), and were also associated with resistance to four other antibiotics. In addition to oxacillin, they are resistant to penicillin, ampicillin, gentamicin and Kanamycin. This high rate of resistance in clinical isolates was reported previously by many authors ; 54% in Egypt(Elsayed *et al.,* 2009) , 57% in Jordan (Al-Zu'bi *et al.,* 2004) 58% in Japan (Murono *et al.,* 2002), 51% in Saudi Arabia (Alghaithy *et al.,* 2000), 61% in

Taiwan (Huang *et al.*,2000), 65% in Kuwait (Gerberding *et al.*,1991) and 69.4% in one report and 78.0% in another report from Sudan (Maimona *et al.*, 2014 ; Omar *et al.*, 2014).

The presence of the *mec*A gene is considered the hallmark for identification of MRSA strains (Kumurya, 2013). This statement was approved by many researchers all over the world: in Sudan (Maimona *et al.*, 2014; Omar *et al.*, 2014), England (Hartman *et al.*, 1984; Wongwanich *et al.*, 2000; Al Zahrani, 2011), Iran (Fateh Rahimi *et al.*, 2013), in Iraq(Al-Abbas, 2012), Egypt (Elsayed *et al.*, 2009), Canada (Farrell, 1999), Japan (Hotta *et al.*, 2000), Australia (Cloney *et al.*, 1999), Spain (Del-Valle *et al.*, 1999), Saudi Arabia (Al-Khulaifi *et al.*, 2009) and in in Jordan(Al-Zu'bi *et al.*, 2004). Moreover, the *mec*A gene was detected in 98% of clinical MRSA isolates from the USA (Al-Abbas, 2012). However the findings in the present study suggested low frequency of the *mec*A gene (68%), this may open the door to search for other intrinsic factors that may compete *mecA* gene in producing resistance phenomenon in regions with high prevalence of MRSA. The absence of *mec*A in MRSA strains has been reported by many authors worldwide (Hawraa *et al.*, 2014; Murakami *et al.*, 1991; Bignardi *et al.*, 1996; Chambers *et al.*, 1989; Ligozzi *et al.*, 1991). Also clinical strains without *mec*A and with methicillin MICs in the 4-16 mg/L range (low dose) have also been reported (Hiramatsu *et al.*, 1992; Geha *et al.*, 1994). A previous study in Nigeria reported the complete absence of five major SCC*mec* types and *mec*A genes as well as the gene product of PBP2a in isolates which were phenotypically MRSA suggesting a probability of hyper-production of β-lactamase as a cause of the phenomenon (Baird, 1996). Moreover, a recent study identified a number of amino acid substitutions present in the endogenous PBPs 1, 2 and 3 that in the resistant isolates which maybe the basis of resistance suggesting that resistant MRSA could be

misdiagnosed using molecular methods alone and draw attention to consider alternative mechanisms for β-lactam resistance in MRSA(Bertrand *et al.*, 2000).

Infections caused by vancomycin-resistant *S. aureus* have been associated with high morbidity and mortality rates. VRSA is one of the common causes of hospital-acquired infections (Anupurba *et al.*, 2003). The glycopeptide vancomycin was considered to be the best alternative for the treatment of multi drug resistant MRSA (Wootton *et al.*, 2001). Vancomycin is the main antimicrobial agent available to treat serious infections with MRSA but unfortunately, decrease in vancomycin susceptibility of *S. aureus* and isolation of vancomycin-intermediate and resistant *S. aureus* have recently been reported from many countries (Benjamin *et al.*, 2010), initially vancomycin-intermediate *S. aureus* (VISA) noted in Japan in 1996 and subsequently in United States in 1997, was believed to be due to the thickened cell wall (Cui *et al.*, 2006), where many vancomycin molecules were trapped within the cell wall. The trapped molecules clog the peptidoglycan meshwork and finally form a physical barrier towards further incoming vancomycin molecules (Cui *et al.*, 2006). Subsequent isolation of VISA and VRSA isolates from other countries including Brazil (Oliveira *et al.*, 2001), France (Poly *et al.*,1998), United Kingdom(Howe *et al.*, 1998), India (Tiwari and Sen, 2006; Assadullah *et al.*, 2003) and Belgium(Pierard *et al.*,2004) has confirmed that the emergence of these strains is a global issue.

The present study showed clearly the existence of VRSA (6.5%) among the enrolled subjects, these findings were suggested previously in Sudan by El imam *et al* (2014) and Ahmed *et al* (2014), in USA by Rohan *et al* (2010), in India by Bhateja *et al* (2005) , Hare and Malay (2006) and in Neigeria (Ilang Donatus *et al.*, 2013). Our findings may be in contrast to the most work conducting in this

field and this may be attributed to abuse of antibiotics and lacking of local regulations and policies which control the emergence of resistant strains.

The genetic mechanism of vancomycin resistance in VRSA is not well understood. Several genes have been proposed as being involved in certain clinical VRSA strains (Jansen *et al.*, 2007; Maki *et al.*, 2004).

In this study, all the VRSA isolates carry *mec*A, but only three contained *van*B. This may open the door to the researchers in this field to seek for other factors which may be responsible for VRSA phenomenon rather than *van*s genes.

Sequencing was used to evaluate not only resistance but also for the investigation of epidemiologic markers. In addition, it is a good tool for determining the evolution of the studied organism as well as for studying one or more genes inside the same organism. Here sequencing was performed for all *mec*A and *van*B gene to determine any mutations if present. The alignment with the published sequence in GenBank was also performed. Sequencing analysis revealed that no obvious mutations were detected among all isolates.

CHAPTER SIX

Conclusion and Recommendations

6.1 Conclusion

In conclusion, the results indicate the high prevalence of multidrug-resistant MRSA in patients (61.5%), this revealed an alarming mark for difficulty to control these bacteria in future putting into consideration the emergence of VRSA strains. Also PCR- based techniques for the detection of all genes responsible for the resistance of *S. aureus* infections is highly recommended to have an obvious idea about genetic variation in this important pathogen.

While VRSA was present with high frequency (4%), putting into consideration the difficulties facing the detection of vancomycin resistance in clinical microbiology laboratory, it recommended to follow the CDC policy which adopted three criteria to identify VISA strains. Broth microdilution vancomycin MIC of 8-16μg/ mL, E-test vancomycin MIC of >6μg/mL and growth on BHI agar containing 6μg/mL vancomycin within 24 hours.

This study demonstrates that only *van*B can be use as diagnostic tool for VRSA strains. This finding has important implications for the management and controlling outbreak and emerges of VRSA in Shendi community. On the basis of this finding, attention should also be given when using conventional disk diffusion method when evaluating resistant *S. aureus* isolates.

Sequencing analysis of *arc*C and *mec*A genes and blasting of their structure nucleotides with the data bank appeared with no significant deviation or mutation.

6.2 Recommendations

1. Further organized surveillance is needed in order to reliably determine the prevalence of MRSA and VRSA in Shendi City as well in Sudan.
2. An effort should be made with governmental authorities to control abuse of antibiotics and prevention of the emergence of resistant strains.
3. The study suggests that a well established laboratory in countries such as Sudan with high burdens of MDR should consider the use of PCR in combination with conventional techniques to enhance prompt and précised detection of VRSA and MRSA among the community.
4. It is recommended to use multiplex PCR in further studies to increase quantity and specificity detecting gene responsible of MDR pathogenes.

Further studies must consider different genes rather than *van*A and *van*B when searching for other factors responsible for VRSA phenomenon

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Appendix I

Questionnaire

1.0 Patient Details

1.1 Date of birth __ /__ /____

1.2 Gender M□ F□

1.3 Patient hospital number: ____

2.0 Details of Hospitalization

2.1 Name of hospital: ----------

2.2 Date of admission ------/------/------

2.3 Patient's primary diagnosis: ----------

Ward/ Unit name: ----------

3.0 Details of infection

3.1 What type of skin infection? Surgical catheter other

3.2 Why was the culture done?

3.3 Is it the first time?

3.4 Did the patient use of antibiotic in past six months?

Antibiotics given for the first episode:

Antibiotics given	date started	Duration of treat.	Route of administration
----------	----------	----------	----------

Hospital admission in past six months Y□ N□

3.5 Please provide details of antibiotic treatment given now:

Culture date: reason for culture:Sensitive antibiotics: Resistant antibiotics:

Antibiotic Given	Date Started	Duration of Treatment	Route of Administration
1. _____	__/__/__	_____	
2. _____	__/__/__	_____	

3. _____ __/__/__ _____

4.0 Invasive Procedures

4.1 Did the patient undergo any of the following invasive procedure(s)

Vascular/ Cardiac Catheterization □ Surgery□ Endosopy□ Urinary catheter □

IV line□ feeding tube□ tracheostomy□ dialysis access □

If "Other", please specify: ----------

Or in the previous 12 months----------

5.0Personal hygiene factors

5.1# Shower /week <6 □ 7 □ >7 □

5.2Shared room or contact with implicated health care worker or another patient Y□ N□

5.3Sharing personal items: Cosmetics □ shampoo □ powder □

nail clipper □ Shaver or razors □ bedding □ laundry □

etc----------

6.0Patient's Social practices:

6.1Gym membership Y□ N□

6.2Participation in sports group Y□ N□

6.3Primitive barbershop Y□ N□

6.4Overweight Y□ N□

6.5Visit prison in previous 12 months Y□ N□

7.0History of patient's health:

7.2 Was the patient or his family hospitalized in previous 12 months? Y□ N□

7.3 History of diabetes Y□ N□

7.4 History of any chronic or acute illness Y□ N□

7.5 History of hospitalization: surgery within 18 months□

Dialysis □ nursing home □

7.6 History of carrying the same illness: family or friend in past 12 months Y□ N□

7.7 Did the patient have any wound didn't heal before? Y□ N□

Appendix II

Preparation of Media

1. Blood agar (Scharlau, Union)

Nutrient agar was used as basal media and was prepared according to instruction of manufacture, after sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes, the media was cooled to 50-55, then 5-10% of blood was added to the basal media and 20-25 ml of molten preparation were poured into sterile disposable 90 mm in diameter Petri dishes, (Mackie and McCartney, 1996).

2. MacConkey Agar

2.1 Formula of MacConkey's Agar (pH 7.1) (Mast Diagnostic, UK)

Selected peptone mixture..... 17.0 g

Lactose..... 10.0 g

Bile sales .....0.5 g

Sodium chloride ..... 5.0 g

Neutral red..... 0.05 g

Agar A..... 16.0 g

Distilled water ..... 1,000 ml

2.2 Preparation

Media was prepared according to instruction of manufacture as follow. 49.5 grams were suspended in 1000 ml distilled water. The preparation heated to boiling to dissolve the medium completely, then sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes. After cooling to 50-55 ^{o}C, and 20-25 mL of molten preparation were poured into sterile disposable 90 mm in diameter Petri dishes, (Mackie and McCartney, 1996).

3. Mannitol Salt Agar Media

3.1 Formula of Mannitol salt Agar Media (pH 7.3) (Oxoid, United Kingdom)

Lab-lemco powder……..1.0

Peptone……...10.0

Mannitol…….10.0

Sodium chloride……75.0

Phenol red……..0.025

Agar……15.0

3.2 Preparation

Media was prepared according to instruction of manufacture as follow. 11.1 grams were suspended in 100ml distilled water. The preparation heated to boiling to dissolve the medium completely, then sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes. After cooling to 50-55, and 20-25 mL of molten preparation were poured into sterile disposable 90 mm in diameter Petri dishes. Date the medium and give it a batch number (Mackie and McCartney, 1996).

4. Tryptone Soya Broth

4.1 Formula of Tryptone Soya Broth (oxoid)

Pancreatic digest of casein..... 17.0 g

Papic digest of soybean meal..... 3.0 g

Dextrose ..... 2.5 g

Sodium chloride ..... 5.0 g

Dipotassium phosphate..... 2.5 g

Distilled water ..... 1,000 ml

4.2 Preparation

30.0 grams was suspended in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Then it was distributed into final containers. Finally sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes

To prepare preserved media with 20 % of glycerol

Add 20 ml of glycerol to 80 ml of media. Distribute 1.5 ml into autoclavable eppendroff tubes. Sterilize by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes (Mackie and McCartney, 1996).

5. Mueller-Hinton Agar (HIMEDIA, India)

5.1 Formula of Mueller-Hinton Agar (pH 7.4) formula

Beef, infusion..... 300.0 g

Cas amino acids..... 17.5 g

Starch..... 1.5 g

Agar..... 17.0 g

Distilled water ..... 1,000.0 ml

5.2 Preparation

Suspend 38.0 grams in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes. Mix well before pouring.

Appendix III

Reagents and Satins

1. Gram's Stain

Most bacteria can be differentiated by their Gram reaction due to differences in the cell wall structure into Gram positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol, and Gram negative which after being stained with crystal violet lose their color when treated with acetone or ethanol, and stain red with Safranine.

1.1 Required

1- Crystal violet stain HiMedia
2- Lugol's iodine HiMedia
3- Acetone HiMedia
4- Safranin HiMedia

1.2 Method of Preparation

1- The dried smear was fixed by heat or alcohol.
2- The fixed smear was covered with crystal violet for 30-60 minutes
3- The stain was washed off with clean water
4- Tip of all water, and the smear covered with Lugol's iodine for 30-60 minutes
5- The stain was washed off with clean water
6- Acetone was rapidly applied (few seconds) for decolourization, and then washed rapidly with clean water
7- The smear then was covered with safranin stain for 2 minutes
8- The stain was washed off with clean water, and wipe the back of the slide clean
9- After air-dry the smear was examined microscopically using immersion oil lens.

1.3 Results

Gram positive .....Dark purple

Stapylococcus aureus appear as cluster forming (grape like) gram positive cocci.

Gram negative .....Pale to dark red

Gram negative *Enterobacteriaceae* and *Pseudomonas* appear as gram negative rod.

2. Catalase Test

2.1 Requirements

Hydrogen peroxide 3% H_2O_2

2.2 Method

1- Pour 2–3 ml of the hydrogen peroxide solution into a test tube.
2- Using a sterile wooden stick or a glass rod, remove several colonies of the test organism and immerse in the hydrogen peroxide solution.
3- Look for immediate bubbling

2.3 Result

Active bubbling . . . . . Positive catalase test
No bubbles . . . . . Negative catalase test

3. Coagulase test

Slide test to detect bound coagulase

3.1 Requirement

Undiluted human plasma (pooled)

Slide test method (detects bound coagulase)

1- Place a drop of distilled water on a slide.
2- Emulsify a colony of the test to make thick suspension.
3- Add a loopful of plasma to one of the suspensions, and mix gently. Look for clumping of the organisms within 10 seconds.

Results

Clumping within 10 sec. . . . . Positive test
No clumping within 10 sec. . . . . Negative test

3.2 Method Tube Test to Detect Free Coagulase

1- Diluted plasma was prepared (1in10 physiological saline) by mixing 0.2 ml of plasma with 1.8 ml of saline.
2- Add 0.8 ml of the test broth culture.
3- After mixing gently, incubate the three tubes at 35–37 ^{0}C. Examine for clotting after 1 hour. If no clotting has occurred, examine after 3 hours. If the test is still negative, leave the tube at room temperature overnight and examine again.

3.3 Results

Clotting of tube contents . . . . . Positive test
No clotting or fibrin clot . . . . Negative test

4. Preparation of Turbidity Standard

1- 1 % v/v solution of sulpharic acid was prepared by adding 1 ml of concentrated sulpharic acid to 99 ml of water. Mix well

2- 1.17% w/v solution of barium chloride was prepared by dissolving of 2.35g of dehydrate barium chloride ($Bacl_2.2H_2O$) in 200ml of distilled water.

3- To make the turbidity standard 0.5 ml of barium chloride solution was added to 99.4 ml of the sulpharic acid solution. Mix well

4- A small volume of the turbid solution was transferred to screw-caped bottle of the some type as used for preparing the test and control inoculate (Mackie and McCartney, 1996).

5. Antibiotics Discs used for Double disc Diffusion Test (Standardized Test)

No.	Antibiotics	Potency	Symbol	Source
1	**Penicillin G**	10 units	**P**	Axiom
2	**Methicillin**	5 mcg	**ME**	Himedia
3	**Cefotaxime**	30 mcg	**CF**	Axiom
4	**Ceftriaxone**	30 mcg	**Ctr**	Oxoid
5	**Cefuroxime**	30 mcg	**CXM**	Pharma
6	**Cephalexine**	30 mcg	**PR**	Axiom
7	**Ciprofloxacin**	5 mcg	**CP**	Axiom
8	**Clindamycin**	2 mcg	**CD**	Axiom
9	**Cloxacillin**	5 mcg	**CX**	Axiom
10	**Co- Trimoxazole**	25 mcg	**BA**	Axiom
11	**Erythromycin**	15 mcg	**E**	Axiom
12	**Gentamycin**	10 mcg	**G**	Axiom
13	**Ofloxacin**	5 mcg	**OF**	Axiom
14	**Pefloxacin**	10 mcg	**PF**	Axiom
15	**Tetracycilin**	30 mcg	**T**	Axiom
16	**Vancomycin**	30 mcg	**V**	Himedia

Appendix IV

PCR Reagents

Gel electrophoresis reagents

Buffers preparation:

10X TBE buffer:

Tris base	108g	0.89M
Boric acid	55g	0.89M
0.5M EDTA	4.6g	20Mm

pH 8.3

dH_2O 1000ml

Preparation 1.5% agarose gel preparation for genomic DNA

0.75g was completely dissolved in 50 ml 1X running buffer by heating, the melted gel then was allowed to cooled to 50 C^o, where 3 drops of ethidium bromide (10mg\ml) was added to melted agarose

Ethidium bromide

Ethidium bromide was prepared by dissolving 1g of ethidium bromide in 100ml distilled water and was kept in brown bottle.

Loading dye: Was prepared by mixing

30% v/v glycerol,

25% w/v bromophenol blue

25% w/v xylene cynol.

Store in 4 C^0 to avoid mold growing in sucrose.

Gel electrophoresis procedure

The melted agarose containing ethidium bromide was poured into gel electrophoresis running tank and was allowed to solidify. The comb was removed and 1X running buffer was added. 2µl of loading dye was mixed with 1µl of DNA marker. The mixture was then placed in the gel well. To each of others gel wells a mixture containing equal volume of loading dye and amplified genomic DNA was applied. The electrophoresis tank was connected to power supply and switch

on and the voltage was adjusted to 75V for 30 minutes. Following gel running the bands of DNA was visualized by using ultraviolet light in gel documentation system.

PCR reaction buffer (-10X standard reaction buffer (100 mM Tri-HCl pH8.5, 500Mm KCl, 15 Mm $MgCl_{2,}$1% Triton X-100)

$MgCl_2$ 25mM

dNTPase Nucleotides(mixed) (100mM 0.8 ml)

Tag DNA polymerase

DNA (marker) ladder size rang (100—3.000 bp) New England biolab

PCR equipments

Power supply blue power 500 SERVA.

Sigma centrifuge 1---15 GERMANY.

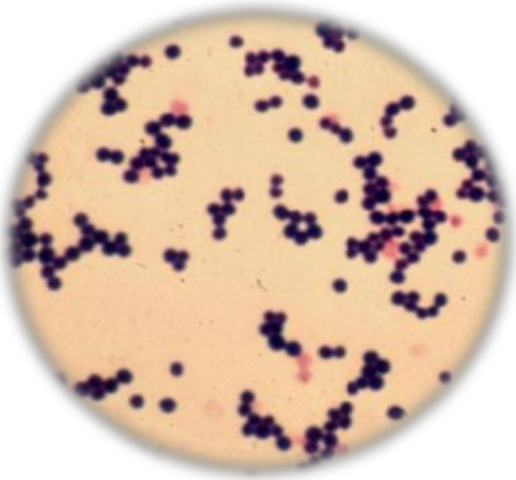
Water bath scott science UK.

PCR machine technique.

Synger gel decommentation system, synoptiesl LTD, UK.

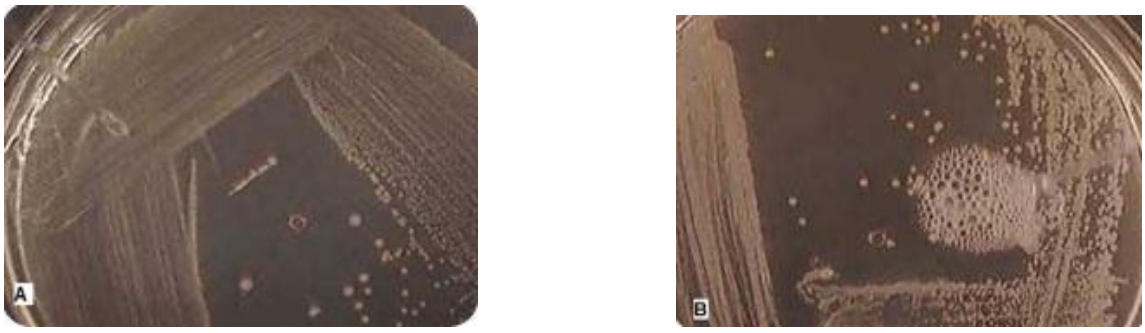
Appendix V

Colored Plates

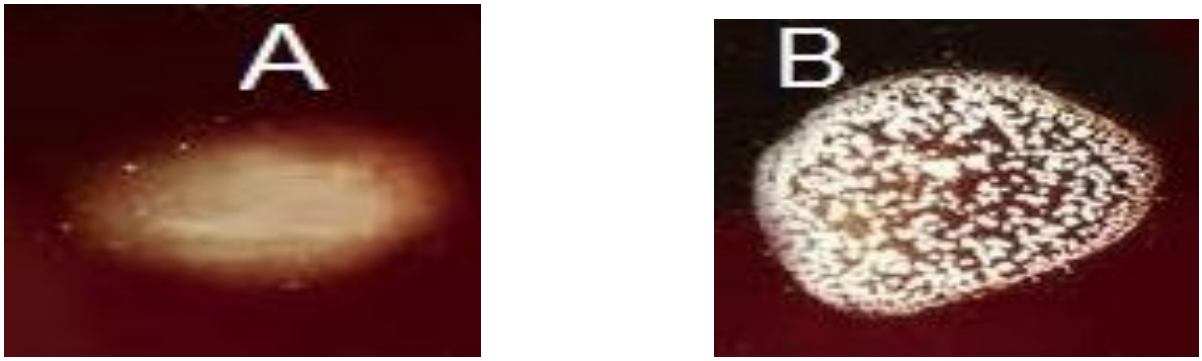


Color plate 1. *Staphylococcus aureus* under microscope with X100 objectives

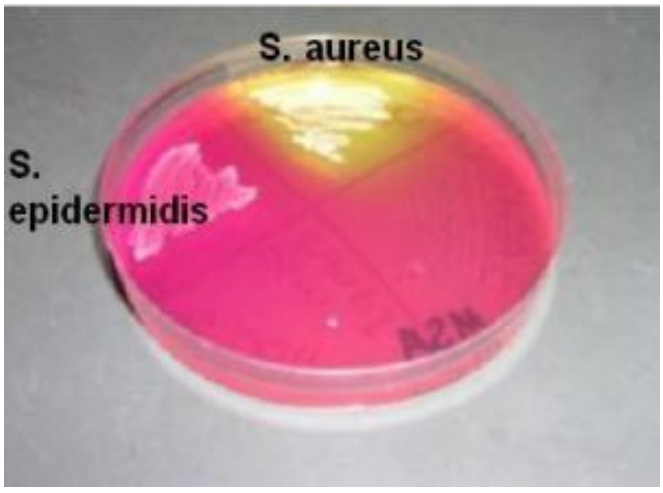
Color plate 2. Overnight growth of *Staph*. *aureus* on Blood Agar medium which produces yellow color



Color plate 3. Catalase reaction; A: negative reaction, B: positive reaction

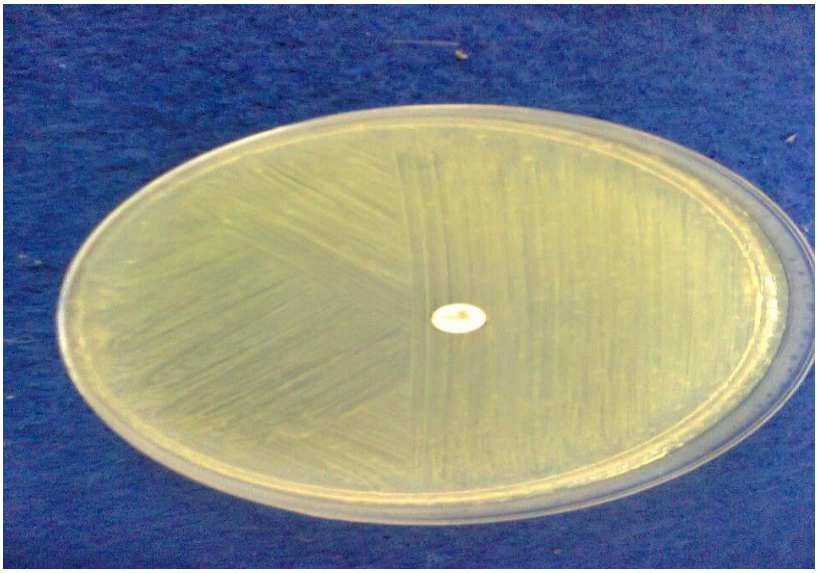


Color plate 4. Slide coagulase test; A: negative reaction, B: positive reaction



Color plate 5. Fermentation reaction of *Staph. aureus* on MSA medium

Color plate 6. Growth of *Staph. aureus* on DNase medium showing positive result with clear zone area around the colonies.



Color plate 7. DST MRSA

Color plate 8. DST VRSA

إستمارات الموافقة الأخلاقية

استمارة موافقة المريض على المشاركة بالبحث

يجب أن تكتب الاستمارة بلغة عربية واضحة ومفهومة وتحتوي على الفقرات الآتية

أسم الباحث.....

عنوان الباحث.....

مكان إجراء البحث.....

أنت مدعو للمشاركة ببحث علمي سريري في (.....).

يرجى أن تأخذ الوقت المناسب لقراءة المعلومات الآتية بتأنٍ قبل أن تقرر إذا ما كنت راغباً بالمشاركة أم لا. وبإمكانك طلب مزيداً من

الإيضاحات أو المعلومات الإضافية عن أي أمر مذكور بالاستمارة أو عن الدراسة من طبيبك.

1- وصف مشروع البحث وأهدافه ومساره:
2- الفوائد الايجابية المحتملة للمشارك التي قد تنتج من هذا البحث.
3- التأثيرات السلبية أو الأعراض الجانبية المحتملة التي يتعرض لها المشارك.

وفي حال موافقتك على المشاركة في هذه الدراسة سيبقى أسمك قيد الكتمان. ولا يسمح لأي شخص حق الاطلاع على الملف الطبي الخاص بك .

وثيقة الموافقة التحريرية

لقد أوضحت للمشارك بالتفصيل البحث وطبيعته ومجرياته وفوائده المحتملة وسلبياته المحتملة أيضاً. وأجبت عن كل استفساراته وأسئلته بوضوح . وسأعلم المشارك بأي تغييرات في مجريات البحث أو فوائده أو سلبياته حال حصولها في أثناء البحث.

أسم الباحث ..... التوقيع التاريخ / /

موافقة المشارك

لقد اطلعت على استمارة الموافقة وأدركت مضمونها وتمت الإجابة عن جميع الأسئلة التي تجول في ذهني. وبناءً عليه فأني حراً ومختاراً أوافق على المشاركة بالبحث. وفهمت أن الباحث / الدكتور ( ) وزملاؤه ومساعديه سيكونون مستعدين للإجابة عن أسئلتي المستقبلية. وباستطاعتي الاتصال بهم على رقم الهاتف ( ). كما أعلم تماماً بأنني حر في الانسحاب من هذا البحث متى شئت ولو بعد الموافقة التحريرية ومصادقتها من دون أن يؤثر ذلك على العناية الطبية المقدمة لي. وسيزودني الباحث بنسخة من هذه الموافقة الخطية.

أسم المشارك..... التوقيع التاريخ / /

بسم الله الرحمن الرحيم

جامعة شندي

مستشفى الملك نمر الجامعي

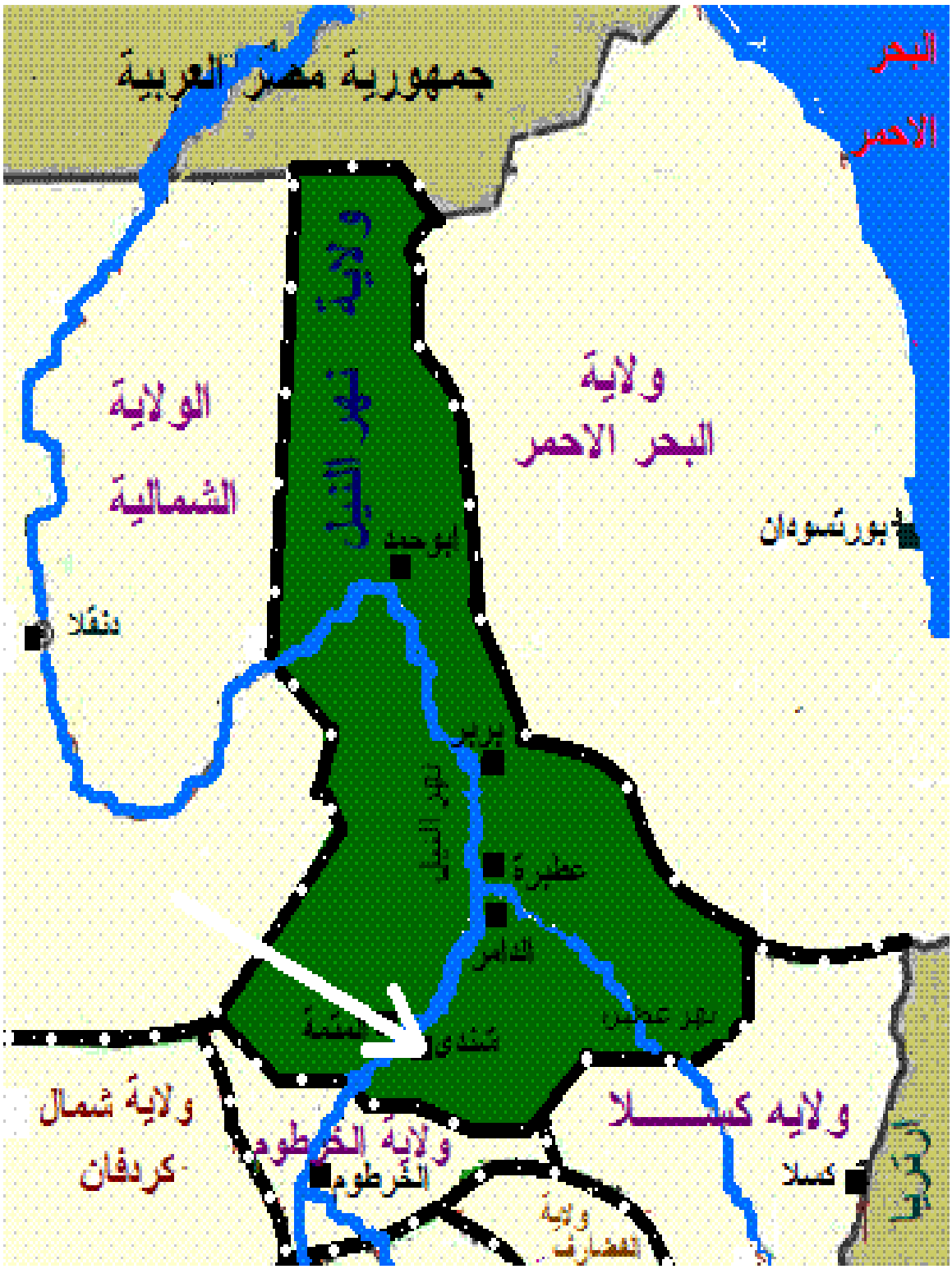
الموضوع / موافقة المؤسسة الصحية على إجراء البحث

بالإشارة للموضوع اعلاه وبعد الاطلاع علي البحث المقدم من الطالب / **محمد احمد التجاني** وان إجراء البحث ليس له اي تاثيرات جانبية علي المرضي لذلك لا مانع لإدارة المستشفي من أخذ العينات من المرضى شريطة اخذ موافقتهم بعد شرح تفاصيله الكاملة وفوائده .

اسم مدير المستشفي..... التوقيع .....

التاريخ :٥/ ٩/ ٢٠١١م

جامعة شندي
التاريخ
٢٠١١/٩/٥
مستشفى الملك نمر الجامعي


جمهورية مصر العربية
البحر الاحمر
ولاية البحر الاحمر
الولاية الشمالية
ابوحمد
بورتسودان
دنقلا
بربر
عطبرة
الدامر
بحر عطبرة
شندي
سنار
ولاية شمال كردفان
ولاية الخرطوم
الخرطوم
ولاية القضارف
ولايه كسلا
كسلا