Sudan University of Science and Technology

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Detection of *Cna, Ica, Hlg* and *SdrE Virulent genes from Staphylococcus aureus* Isolates in Khartoum State

التعرف على جينات *Cna, Ica, Hlg* and *SdrE* الضارية في معزولات المكورات العنقودية في ولاية الخرطوم

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الآيـــــة

بِسْمِ اللّهِ الرَّحْمَنِ الرَّحِيمِ

قُلْ أَعُوذُ بِرَبِّ الْفَلَقِ (1) مِنْ شَرِّ مَا خَلَقَ (2) وَمِنْ شَرِّ غَاسِقٍ إِذَا وَقَبَ (3)

وَمِنْ شَرِّ النَّفَّاثَاتِ فِي الْعُقَدِ (4) وَمِنْ شَرِّ حَاسِدٍ إِذَا حَسَدَ (5)

سورة الفلق

DEDICATION

The research work is dedicated to the beloved ones; my father, my mother and my family members.

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I thank the Almighty Allah for making it possible for me to complete this work successfully. I also need to stress out my heartfelt gratitude to my supervisor Prof. Yousif Fadlallah Hamedelnil, Dr. Hisham Altayeb as well as my colleague Mr. Hossam for their unlimited support.

ABSTRACT

S. aureus is an extraordinarily versatile pathogen, and it can cause a large spectrum of infections, from mild to severe and fatal. It is important in humans and also economically important when infecting animals, able to cause superficial lesions and systemic infections. The aim of this study is to detect *Can, Ica, Hlg* and *SdrE* virulent genes of *Staphylococcus aureus* associated with different types of infections among Sudanese in Khartoum State. The Samples were collected from different hospitals in Khartoum, and participants were of all ages who visited hospitals for care or for treatment of different illnesses. Sixty five isolates were confirmed as *Staphylococcus aureu* by primary and secondary biochemical tests. Among which 30 (46%) were from infected wounds followed by urine samples 25 (38.46%) and blood samples 10 (15%). The sensitivity profile of the isolates to Vancomycin, Gentamicin and Ciprofloxacin showed that more than 50% of the isolates are resistant to these antibiotics. *Ica* gene was found predominating (73.85%) of the isolates. *Sdr*E 38.46% and *Can* and *Hlg* genes were 29.25% and 7.69% respectively. The relationship between the virulence genes and resistance to antibiotics showed that the highest resistance was observed in isolates with *Ica* and *SdrE* followed by *Cna* and *Hlg*, The relationship between virulent genes and antibiotic resistance was indicated significant relationships (p=.03) between Ciprofloxacin resistance and the presence of *SdrE* gene as well as between Methicillin resistance and the presence of *SdrE* and *Ica* genes (p= .00 for both). Distribution of virulent genes according to gender and age indicate no significant associations in any *Staphylocoocus aureus* virulent genes

in the current study with age or gender. Regarding type of samples; significant association (p= .00) has been detected between *Hlg* gene and the type of sample, and no other significant relationships were detected. Information generated from the current study can be used by surveillance agencies to identify the new strains, link one type of infection to other or identify emerging or re-emerging strains that may have significance in causing outbreaks.

ملخص الاطروحة

باكتيريا المكورة العنقودية معروفة في كونها مسببة لعدد كبير من الأمراض متفاوتة الضراوة, مهمة لصحة الانسان وكذلك ذات اهمية اقتصادية عند اصابة الحيوان. مسببة للالتهابات الجلدية وكذلك الامراض العامة. هدفت هذه الدراسة للتعرف على جينات (*Can, Ica, Hlg* and *SdrE*) الضارية للمكورات العنقودية الذهبية والمسببة لامراض مختلفة بين السودانيين في ولاية الخرطوم. تم جمع العينات من مستشفيات مختلفة في ولاية الخرطوم, المشاركون في الدراسة كانو مرضى قاموا بزيارة مستشفى للعلاج من امراض مختلفة. 65 من العدد الكلي للمشاركين وجدت بعيناتهم باكتيريا المكورات العنقودية الذهبية.30 عينة 46% من العينات كانت من جروح ملتهبة, 25 عينة 38,46% كانت من عينات بول و 10 عينات 15,38% كانت من عينات دم. ملف الحساسية أظهرأن اكثر من 50% من الباكتيريا المعزولة مقاومة لمضادات الفانكومايسن, الجنتامايسين والسيبروفلوكساسين. جين (*Ica*) كان الاكثر وجودا في العينات تحت الدراسة 73,85% من العينات, جين (*Sdr*) بنسبة 38,46%. جيني *Can* و *Hlg* كانوا 29,52% و 7,69% على التوالي. العلاقة بين الجينات الضارية والمقاومة للمضادرات الحيوية أظهرت أن اعلى مقاومة ظهرت في الباكتيريا المحتوية على جيني *Ica* و *SdrE* يليهما جيني *Cna* و *Hlg*, العلاقة بين الجينات الضارية والمقاومة للمضادات الحيوية تشير الى علاقة بين المقاومة لمضاد السيبروفلوكساسين ووجود جين *SdrE* (P= .03), اضافة لمضاد الميثيسيلين وجيني *SdrE* و *Ica* (P=.00). علاقة وجود الجينات الضارية بالجنس والعمر لم يظهر اي علاقة جادة. بالنسبة لنوع العينة, هناك علاقة (P=.00) بين جين (*Hlg*) ونوع العينة, ولم يتم ايجاد اي علاقة اخرى. المعلومات الناتجه من هذه الدراسة من الممكن استخدامها للجهات المعنية بالمسح في هذا المجال ليتم التعرف على طائفة جديدة, الربط بين مرضين مختلفين أو التعرف على طوائف ناشئة أو نشأت من جديد مرتبطة بانتشار وباء جديد.

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Chapter One

Introduction

Chapter One

Introduction

1.1.1. Background

Approximately half of the recognized *Staphylococci*are known to inhabit the human body, with particular strains showing specificity for defined regions of the body (Longauerova, 2006; Piette & Verschraegen, 2009). *S. epidermidis* and *S. hominis* are the most abundant *Staphylococci*on the trunk and limbs respectively (Gemmell, 1986), *S. capitis* on the head and *S. auricularis* on the external ear (Von Eiff *et al*., 2002). A heightened recognition of the importance of *Staphylococci* in nosocomial infections has come about through the increased use of indwelling devices. *Staphylococci*are now known to be responsible for several diseases, including pneumonia, endocarditis and osteomyelitis and are the most common cause of venous catheter-related bacteraemia, 50-70% of cases are caused by *S. epidermidis* (Casey *et al*., 2007; von Eiff *et al*., 2002).

The bacteria are able to resist the low pH and high osmolarity of the skin and possess extracellular enzymes including fatty acid modifying enzyme (*FAME*) and other virulence factors, including adhesins, phenol soluble modulins (*PSMs*) and some strains produce haemolysin (Longauerova, 2006). Biofilm formation allows growth of *Staphylococci*bacteria on indwelling devices, after initial adhesin-mediated attachment (Piette & Verschraegen, 2009). Treatment of *Staphylococci* infections is problematic due to the prevalence of antibiotic resistance (von Eiff *et al*., 2002). Information managed to be generated from the current study can be used by surveillance agencies to identify new strains, link one type of infection to other or identify emerging or re-emerging strains that may have significance in causing an outbreak (McLauchlin *et al*., 2000).

1.1.2. Rationale

Staphylococcus aureus is acommensal and lives on the anatomical locales of humans and animals. Sensitivedetection methodologies for genes responsible of pathogenecity are important because virulent genes are potentpathological determinants and the research status in this area in Sudan emphasizes the need for more enforcement. Moreover, detection ofvirulent genes of *S. aureus* is also important for epidemiological reasons.

1.1.3. Study Objectives

1.1.3.1. General Objective

Molecular Detection of *Staphylococcus aureusIca*, *Cna*, *Hlg* and *SdrE* virulence genes isolated from different clinical samples from Sudanese in Khartoum State.

1.1.3.2. Specific Objectives

1. To identify the isolates by primary and secondary bacterial techniques including biochemical tests.

2. To detect 16s rDNA gene from the isolates.

3. To detect *Ica*, *Cna*, *Hlg* and *SdrE* virulence genes using multiplex PCR among different groups of age, gender and source.

3. To determinethe sensitivity of isolates to certain antibiotic according to their virulent genes and correlate between them.

Chapter Two

Literature Review

Chapter Two

LITERATURE REVIEW

2.1. Staphylococcus

The genus *Staphylococcus* is a member of the *Staphylococcaceae* family; they are Gram-positive, cocci arranged in a grape like cluster, facultative anaerobic, chemoorganotrophic cocci with a respiratory and fermentative metabolism at an optimal temperature of 37°C, also, they are non-motile, nonsporulated, catalase positive and found as pathogens or commensal organisms in both humans and animals (Argudin*et al* ., 2010).

There are 47 known species and 24 subspecies in the *Staphylococcus* genus. Approximately half of the species are endogenous to human beings, including *S. aureus* (a coagulase positive species) and coagulase-negative species: *S. epidermidis, S. haemolyticus, S. saprophyticus, S. cohnii, S. xylosus, S. lugdunensis, and S. schleiferi, S. capitis, S. warneri, S. hominis, S. simulans* (Kloos and Schleifer, 1975), *S. saccharolyticus, S. auricularis* (Kilpper-Balz and Schleifer, 1981), *S. caprae* (Devriese *et al*., 1983) . There are also some subspecies that are endogenous to humans and other primates, such as *S. capitis* subsp. *Ureolyticus* (Bannerman and Kloos, 1991) and *S. cohnii* subsp. *urealyticum* (Kloos and Wolfshohl, 1991).

These organisms are resistant to adverse environmental conditions and can be recovered from non-physiological environments even months after inoculation, a peculiar characteristic of staphylococci is their capacity to grow in high salt concentrations, and most of them grow in media with 10% of NaCl (Hennekinne *et al*., 2010).

The species in the genus are classified based on the production of enzyme coagulase. Coagulase production capacity divides staphylococci into two major groups: coagulase positive, including species *S.aureus, S. intermedius, S. schleiferi* subsp. *coagulans* and *S. delphini*; and coagulase negative, including more than 30 different species, species *S.*

hyicus is variably coagulase positive and frequently included among coagulase-negative microorganisms (Cunha, 2009).

2.2. *Staphylococcus aureus*

S. aureus is an extraordinarily versatile pathogen, and it can cause a large spectrum of infections, from mild to severe and fatal. It is important in humans and also economically important when infecting animals, able to cause superficial lesions and systemic infections, *S. aureus* is responsible for toxin-mediated diseases, such as the Toxic Shock Syndrome (TSS), Kawasaki's Syndrome and staphylococcal food poisoning (Leung *et al.*, 1993; Vasconcelos and Cunha, 2010). *S. aureus* is known as one of the most frequent pathogens in both community and nosocomial infections, and it can cause septicemia, endocarditis, osteomyelitis, abscesses, pneumonia, wound infections, impetigo, cutaneous rash, in addition to various toxin-mediated diseases, the variety of such spectrum of clinical manifestations is mostly dependent on the numerous virulence factors produced by each strain (Le Loir *et al*., 2003).

Approximately 30 - 40% of the human population carries *S. aureus*, and its main habitat is the nasopharynx, a site where strains can persist as transitory or persistent members of the normal microbiota without causing any symptoms (Partida *et al.*, 2010).

2.3. Virulence factors

The virulence factors of microorganisms in the *Staphylococcus* genus include surface components, such as the capsule, peptidoglycans, teichoic acid, protein A, collagen cell attachment protein, adhesins, enzymes such as lipases, esterases, fatty-acid modifying enzymes, various proteases, hialuronidase, hydrolytic enzymes, desoxyribonucleases, catalase, betalactamase, staphylokinase, and various toxins, such as enterotoxins, exfoliative toxin A and B, leukocidins, *TSST-1* and alpha, beta, gamma and delta hemolysins (Vasconcelos and Cunha 2010).

Plasma coagulase is an enzyme that functions like thrombin to convert fibrinogen into fibrin tissue, microcolonies surrounded by fibrin walls are difficult to phagocytes, coagulase production is the principal criterion used by the clinical microbiology laboratory for the identification of *Staphylococcus aureus* isolates. Numerous allelic forms of *S. aureus* coagulase exist, with each isolate producing one or more of these enzyme variants (Kayser *et al*., 2005).

The *Coa* gene is one of the most important virulence factors for *S. aureus*, expression of this gene is thought to enhance bacterial growth and promote infection in the face of host defense mechanisms, such as phagocytosis (Karahan, and Cetinkaya 2007).

At least thirty four different extracellular proteins are produced by pathogenic *Staphylococcus* strains, and several of them already play a definite role in the pathogenesis of recognized *staphylococcal* disease (Lisa, 2004). Some genes responsible for such factors are frequently transported by genetic elements, such as phages and pathogenicity islands, these are differently sized and potentially movable DNA segments which encode virulence related genes, and are horizontally transferred among the strains (Yamaguchi *et al*., 2000; Yoshizawa *et al*., 2000).

2.4. Genetic control of virulence determinants

A successful *S. aureus* infection relies on the pathogen's ability to colonize the initialinfection site, adapt to the stresses of the host environment, acquire nutrients for growth,resist host defenses and spread to other sites in the host when the nutrient levels in theoriginal foci of infection are exhausted. To accomplish these stages of infection, *S.aureus* expresses multiple virulence factors from different categories, depending on thestage of infection (Cheung *et al*., 2004; Lowy, 1998). In the initial stage of infection therelatively low numbers of bacteria must attach to host tissues and replicate. *S. aureus*expresses multiple adhesins with specificity to host components of the extracellularmatrix allowing firm binding (Foster, 2005). At this stage the bacteria

mustfend off host immune factors that are present at the site of infection and prevent theingress of additional factors to the site of infection, by producing cell wall-associated orsecreted immune evasion proteins (Foster, 2005). Once the bacterial load has increased,nutrient levels concomitantly decrease and the necessity for immune evasioncomponents is less pressing than the requirement for nutrients. At this stage degradativeenzymes and toxins are produced to allow deeper penetration of tissues and access tonutrients whilst adhesin expression is curtailed to allow detachment and spread (George& Muir, 2007).

2.5. Adhesins

To colonize and persist within its host, *S. aureus* expresses microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Members of the MSCRAMM family allow colonization of wounds (Foster, 2005), skin surface (Coates *et al*., 2014), the nares (Schaffer *et al*., 1997), indwelling devices (Hartford *et al*., 1997) and tissues within the body associated with specific diseases, such as healthy aortic tissue resulting in endocarditis (Hienz *et al*., 1996). The functions of adhesins have been determined by in vitro binding assays using purified protein and the comparison of whole bacteria deficient in a particular adherence protein with the wild type strain. One problem with using purified protein is that unless the protein is expressed along with the posttranslational modification machinery then the effects of posttranslational modifications are not accounted for. Isogenic mutants are also used to assess the importance of a particular adhesin gene to the pathogenicity of *S. aureus*, using in vivo models. These analyses can be problematic due to the high level of redundant function of *S. aureus* adhesins, for instance the extracellular matrix (*ECM*) components fibrinogen and fibronectin are bound by the autolysins *Aaa* and *Atl* (Heilmann *et al*., 2005; Hirschhausen *et al*., 2010), the extracellular adherence protein, *Eap/Map* (Haggar *et al*., 2003), extracellular fibrinogen-binding protein, Efb (Cheng *et al*., 2014; Lee *et al*., 2004), extracellular matrix protein-binding protein and Emp (Hussain *et al*., 2001). These proteins also bind other ECM components and have other roles in infection. This redundancy may well be artefactual and

a result of the methods employed to study *S. aureus* adhesins, or it may be due to differential expression ofadhesins or an evolutionary manifestation of their importance to pathogenicity. Several *S. aureus* adhesins are known to be associated with specific diseases and sites in the host, based on the tissues they promote binding to and in vivo analyses. The collagen-binding protein, *Cna* promotes binding to the cornea promoting the inflammatory eye disease keratitis (Rhem *et al.*, 2000) and is also associated with infective endocarditis by binding to undamaged aortic valves (Hienz *et al.*, 1996).

2.6. Haemolysins

The haemolysins are so named due to their ability to lyse red blood cells (RBCs). Thisfunction is likely important for the acquisition of *Fe*, as ~80% of the body's Fe isassociated with haemoglobin, while most of the remaining is associated as cofactors inother metaloproteins, or cheated by the storage proteins. The alpha, beta and delta haemolysin genes (*hla, hlb and hld*,respectively) are expressed throughout infection, likely due to a need for *Fe*. However,*Hla* mRNA translation to *Hla* is controlled by RNAIII, the agr effector molecule, and*Hla* is encoded centrally on the RNAIII molecule, so *hld* expression must only be at alow level early in infection (Arvidson & Tegmark, 2001; Bronner *et al.*, 2004). Thismay allow for low level expression of *Hla* for the acquisition of *Fe*, which increases asthe need for Fe increases with bacterial load.

Hla is a homoheptameric leukotoxin that interacts with the *ADAM10* receptor on thesurface of endothelial cells. This interaction promotes disruption of the vascularendothelial barrier by inhibiting production of the intercellular adherence proteincadherin (Gouaux *et al.*, 1994; Powers *et al.*, 2012). *Hla* also promotes proinflammatoryIL-1β, IL-18 production in monocytes by promoting formation of theNLRP3 inflammasome complex, a host signalling complex involved in regulatinginflammatory cytokine production. This is followed by necrosis of monocytes in anNLRP3-dependent pyronecrosis, releasing the tissue damaging contents of monocytes(Craven *et al.*, 2009).

2.7.*Sdr*gene

Sdr proteins (from SD Repeat), together with MSCRAMM proteins *ClfA* (clumping factor A) and *ClfB* (clumping factor B), are members of a structurally related family of cell wall anchored proteins. The characteristic feature of the family is the presence of R region containing multiple serine-aspartate repeats (Foster and Hook 1998). The *sdr* locus encodes three proteins, *SdrC*, *SdrD*, and *SdrE*; however, not all three genes are present in all *S. aureus* strains. Also, the transcriptional organization of the region remains unclear. Based on previous analyses (Peacock *et al*.,2002; Sabat *et al*.,2006), it was noticed that the *sdrC* gene is always present in the locus, while *sdrD* and *sdrE* are not. There also seems to be a correlation between carriage/invasive strains and the presence of the *sdrE* gene (Peacock *et al*.,2002). Strains carrying only the *sdrC* gene have a diminished potential to cause bone infections, which may be connected with the fact that one of the allelic variants of *SdrE* was previously identified as a bone sialoprotein-binding protein. *SdrC* binds β-neurexin 1 exodomain and expression of the protein increases adherence to cultured mammalian cells expressing β-neurexin on their surface. Other *Sdr* proteins are involved in adherence to epithelial cells and *SdrD* is crucial in abscess formation.*SdrE* gene is reported to be of 7.3% prevalence among all *S. aureus* isolates(Sabat *et al*., 2006).

2.8. *Hlg* gene

S. aureus produces some bi-component toxins structurally similar to α-toxin. These toxins result from the association of the class S (Slow) component and the class F (Fast) component based on their electrophoretic mobility. They induce the activation and the permeability of the target cells. They can lyse phagocytes (monocytes-macrophages and neutrophils), which is considered important for *S. aureus* immune evasion. These PFTs include the gamma-toxin (gamma-hemolysins *HlgA* and *HlgC/HlgB*) among others like the Panton-Valentine leukocidin (*PVL*).

The *hlg* gene cluster encoding for hemolysin-γ (*Hlg*) and hemolysin-γ2 (*Hlg2*) is located in the core genome. This cluster is present in almost all *S. aureus* strains. These toxins play a role in septic arthritis and could help community-acquired MRSA (CA-MRSA) to survive in human blood during infection (Ventura *et al*., 2010;Dumont*et al*., 2011).

2.9. *Ica* gene

Cell aggregation and biofilm accumulation are mediated by the products of a gene locus composing of the genes *Ica*ACB and D, which encode the essential proteins for the production of polysaccharide intercellular adhesion (PIA) and capsular polysaccharide/adhesion (PS/A) in *Staphylococcus* species. It was demonstrated that the *Ica*-encoded genes are responsible for the biosynthesis of the PIA, which contains N-acetylglucosamine as a main constituent and in the accumulation phase of biofilm formation, playing a crucial role in invasiveness of *S. aureus*. Different studies have shown the decisive role of the *Ica* gene as virulence factors in staphylococcal infections (Hall-Stoodley *et al*., 2004; Frank & Patel, 2007).

2.10. *Cna* gene

Some strains of *Staphylococcus aureus* bind collagenwith a high degree of specificity and affinity. This interaction can represent a mechanism of substrate adhesion and may be an important step in the pathogenesis of infection(Foster and Hook 1998).

2.11. Methods in analysis of virulence factors

2.11.1. Characterization of individual virulence factors *in vitro* and *in vivo*

A frequently used method for the analysis of virulence factors is the disruption ordeletion of a potential virulence factor encoding gene, followed by observation of theresulting phenotype. For an *in vitro* study, this may involve analysis of growth in theabsence of a specific nutrient, or growth on tissue culture or specific host cell type.

Alternatively, the protein product of the putative virulence factor can be overexpressedand purified and then its structural properties and function can be analyzed in isolation.An important problem of *in vitro* analyses is that they do not mimic the hostenvironment well. Inside the host a virulence factor may be released at lowconcentrations, or expressed only at certain times during infection in order to functioncorrectly, while a relatively large amount of purified protein may be used *in vitro* overextended periods (Cross, 2008; Smith, 1998). This may lead to spurious conclusions asto the activity of the virulence factor, such as binding ligands that would not be boundwithin the host at the lower *in vivo* concentration and lower exposure times. In themajority of *in vitro* experiments the media used, including rich media, defined mediaand minimal media is the single source of nutrients throughout the experiment (Quinn *etal*., 1997; Smith, 1998). This does not reflect the conditions *in vivo*, where access tonutrients is changing over the course of infection, which alters the growth rate and geneexpression of the bacteria.

Complementing *in vitro* analyses with *in vivo* studies overcomes many of the mentioned problems above. Putative virulence factor genes from a knownpathogen can be inserted into avirulent or poorly virulent species and the effect can thenbe assessed *in vivo*. RNA interference (RNAi) can be used to knock-down theexpression of pathogen and host gene products, to identify pathogen gene productsrequired for virulence and host products required to fight infection (Powers *et al*., 2012).

2.11.2. Identification of virulence factors by *in silico* analysis

Advances in genome sequencing technologies have led to whole genome sequencing ofmultiple pathogenic species and strains (Buermans & Dunnen, 2014; van Dijk *et al*.,2014). Bioinformatics can be used to compare the genomes of known pathogens toidentify shared virulence factors and compare the host genome with that of the pathogento identify proteins that may interact with the host. Furthermore, whole genomes can besearched for known promoter elements to identify genes controlled by regulators knownto be important for virulence factor expression (Burrack & Higgins, 2007). Availablegenome sequences also allow for ordered transposon mutagenesis libraries of individualstrains, which can be used to assess the importance of non-essential genes to thevirulence of a pathogen (Fey *et al*., 2013). Bioinformatics is a powerful approach thatcan be used to identify potential targets in order to direct work in the laboratory,allowing for a more focused approach to characterize virulence factors.

2.11.3. Selective capture of transcribed sequences (SCOTS)

Selective capture of transcribed sequences (SCOTS) was developed by (Graham &Clark-Curtiss, 1999). The SCOTS method allows comparison of transcribed genes *invivo* with transcribed genes *in vitro*. Total RNA is extracted andconverted to cDNA, then bacterial cDNA (limited to 200-500bp) is captured byhybridization to biotinylated bacterial DNA. The most common cDNA will beribosomal DNA (rDNA), so the biotinylated rDNA site is blocked by an initial round ofhybridization with plasmid DNA encoding rDNA. Subtractive hybridization between linked cDNA from *in vitro* or *in vivo* growth, leaves enriched *invitro* cDNAdemonstrating down-regulation *in vivo*, or enriched *in vivo* cDNA, demonstratingupregulation *in vivo*. SCOTS can be used to determine geneexpression changes *in vitro* using cell cultures (Graham & Clark-Curtiss, 1999) andfrom tissues recovered from *in vivo* animal models (Baltes & Gerlach, 2004). Thismethod can identify genes positively or negatively regulated, but says nothing of thefunction of the genes.

2.11.4. Signature-tagged mutagenesis (STM)

Signature-tagged mutagenesis (STM) allows analysis of multiple genes within a singlehost, to identify those genes required by the pathogen for survival in the host. STM wasdeveloped by(Hensel *et al*.,1995). The basic STM method relies on variable tags, with identical flanking regions,allowing PCR of all the variable regions in a single reaction.

Chapter Three

Materials and Methods

Chapter Three

Materials and Methods

3.1.1. Study design

Descriptive cross sectional study in Kartoum, Sudan.

3.1.2. Study area

The Samples were collected from Omdurman military hospital, Police hospital, Soba hospital and Bahary hospital representing Khartoum State.

3.1.3. Study population

The subjects were of all ages who visited hospitals for care or for treatment of urinary tract infections, wound infections or preceded for blood culture inquiries. Both sexes were eligible for enrolment into the study.

3.1.4. Sample size

Sixty five samples of urine, wound and blood were collected from different hospitals from Khartoum State.

3.1.5. Study duration

For two successive months; from the 1st of April till the 30th of May, 2017, all patients who visited hospitals for urine, wound or blood culture inquiries were recruited to the study after their approval.

3.1.6. Ethical Consideration

All patients (inpatient or outpatient) have been asked to sign consent prior to specimen collection. Verbal consent from laboratory administration has been also provided priorthe collection process.

3.1.7. Data collection

Data was collected using structural questionnaire (Appendix).

3.1.7.1. Laboratory work

3.1.7.1.1. Specimen collection

Isolated bacteria were collected from hospitals and subculturedonto sterile media (Nutrient agar)(Himedia Laboratories Pvt. Ltd, Mumbai 400086, India)for further analysis.

3.1.7.1.2. Cultivation

The bacteria were immediately cultured ontoblood agar and Manitol Salt Agar (Himedia laboratories Ltd, Mumbai 400086, India) in the microbiology laboratory in Faculty of Medical Laboratory Sciences at Sudan University of Science and Technology. Specimens were incubated aerobically for 24 hours at 37°C.

3.1.7.1.3. Bacterial identification

Colonies were examined the next day. The organisms were identified according to the morphology of the colonies; Gram stain and biochemical tests were prepared and examined for the growing organism.*S. aureus* specific 16S rRNA genes were also detected for identification of *S. aureus* (Table 1).

3.1.7.1.3.1. Gram stain

Gram stain which is essential technique for initial identification of bacterial isolates was introduced. The procedure was carried out as illustrated by (Cheesbrough, 2006) in the following procedure; smear was prepared from overnight culture on a clean and dry slide. The smear was left to air dry. Fixation was done by rapid pass of the slide three times through the flame of a Bunsen burner then allowed to cool before staining. Crystal violet stain was added to smear for 30–60 seconds, and then washed by tap water. Lugol’s iodine was added for 30-60 minutes then washed by tap water and decolorized

rapidly (few seconds) with acetone alcohol and washed immediately by tap water. Finally, the smear was covered with saffranin stain for two minutes and washed by tap water. The back of slide was wiped clean and placed in a draining rack for smear to air dry. Drop of oil was added to the dried smear and examined under the light microscope.

All Gram negative organisms were excluded and only Gram positive cocci in clusters were considered. Then biochemical tests (DNAse, catalase, Manitol salt agar and coagulase production tests) were used to identify *Staphyloccus aureus* organisms (Appendix).

3.1.7.1.4. *In Vitro* antibiotic sensitivity testing

Kirby-Bauer method was used in the current study. The antibiotic discs used were from Himedia (Himedia Laboratories Pvt. Ltd, Mumbai 400086, India). Methicciln (5mg), Gentamicin (10mg), Ciprofloxacin (5mg), Vancomycin (30mg) were used (Appendix).

The antibiotic discs were placed onto Muller Hinton Agar(Himedia Laboratories Pvt. Ltd, Mumbai 400086, India). The distance between the two adjacent discs was at least 20 mm and from the edge of the plate was 15 mm. The media were incubated aerobically for 24 hours in 37°C .After 24 hours of incubation the diameter of the zone inhibition was measured and compared with the published tables of the control strains according toCLSI guidelines, (2014).

3.1.7.1.5. DNA Extraction

DNA was extracted by using Guanidine hydrochloride method:

Two ml of buffer ,1ml of Guanidine hydrochloride , 300µl of NH4 acetate and 10 of proteinase K as added to the isolated organism.The samples were incubated overnight at 37°C.After overnight incubation the samples were cooled at room temperature and then 2 ml of pre-chilled chloroform was added after that samples wes centrifuged for 5 minutes at 6000rpm.Upper layer was collected to new falcon tube and 10 ml of cold absolute ethanol was added to the samples then kept at -20 overnight. After overnight

incubation samples were centrifuged for 10 minutes at 6000rpm then the supernatant was drained. Pellet was then washed with 4 ml of 70% ethanol then centrifuged for 10 minutes at 6000rpm.Supernatant was poured off and pellet was allowed to dry.Pallet was dissolve in 100µl of sterile water for injunction and stored at -20. Extracted DNA was determined by running the DNA sample on 1.5% gel agarose (Sambrook *et al.*, 1989).

3.1.7.1.5.6. Preparation of TBE buffer

The following gradients were dissolved in one liter of deionized water for 10x TBE buffer, 108 gm Tris buffer, 55gm of boric acid and 40 ml of 0.5M EDTA. Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved (Sambrook *et al.*, 1989).

3.1.7.1.5.7. Preparation of agarose gel

In 100 ml boiling 1X TBE buffer, 2 grams of agarose was added, 5 µl of (10mg\ml) ethidium bromideswere added to agarose buffer then gently mixed. The mixture was poured onto the casting traywith suitable comb. After solidification, the comb was gently removed (Sambrook *et al.*, 1989).

3.1.7.1.5.8. Virulence gene detection by PCR

Multiplex PCR reactions were carried out using 1 µl DNA solution, 5 µl Qiagen HotStarTaq Master Mix (Qiagen Nr. 203445) and 10 pmol of each gene-specific primer in a final volume of 25 µl. 20 to 30 pmol from each of primers *Hlg, Ica* and *Cna* were used as recommended by (Kumar *et al.*, 2009). This mixture was introduced into multiplex PCR protocol; 40 cycles of (94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, 72°C for 40 seconds and final extinction for 72°C for 5 minutes), origin and expected PCR amplicon sizes are given in [Table 1]. For *SdrE* the following single PCR protocol was conducted: 30 cycles of (94°C for 5 minutes, 94°C for 40

seconds, 50 °C for 40 seconds, 72°C for 40 seconds and final extinction for 72°C for 5 minutes) (Figures 11&12).

Table 1: Details of primers used in the study and amplicon sizes

Primer	Sequence 5' – 3'	Amplicon	Reference
***Cna* - F**	AGTGGTACTACTAATACTG	740	Kumar *et al.*, 2009
***Cna* - R**	CAGGATAGATTGGTTTA		
***Hlg* - F**	GCCAATCCGTTATTAGAAAATGC	937	
***Hlg* - R**	CCATAGACGTAGCAACGGAT		
***Ica* - F**	GATTATGTAATGTGCTTGGA	770	
***Ica* - R**	ACTACTGCTGCGTTAATAAT		
***SdrE* - F**	AGTAAAATGTGTCAAAAGA	767	
***SdrE* - R**	TTGACTACCAGGCTATATC		
16S rRNA-F	AGTTTGATCCTGGCTCAG		
16S rRNA-R	AGGCCCGGGAACGTATTCAC		

3.1.7.1.5.9. Agarose gel electrophoresis

In order to make 2% Agarose Gel; 1.0 gram of agarose was mixed with 50 ml 1x TBE buffer in an Erlenmeyer flask. Heat was applied for 2 minutes using microwave oven. Then it was Left to cool at 50^{o} C and then 2.5 µl of 10 µg/ µl ethidium bromide solution was added. Mixed well and poured in gel pouring chamber. Two combs were then placed in the chamber and left to cool for about 25 minutes. Electrophoresis chamber was then filled with the 1x TBE buffer. Samples and ladder were then loaded to the wells of the gel. Electrophoreses was conducted at 60Volt for 50 minutes (Figure 13).

3.1.7.2. Statistical Analysis

Data were introduced to Scientific Package of Social Sciences (SPSS) software toestimate the *p* value of significance using Chi square test, means and averages were also calculated.

Chapter Four

Results

Chapter Four

Results

From 65 isolates, 30 (46.15%) samples were from infected wounds,25(38.5%)urine samples and 10 (15.4%) blood samples (10) (Table 2). The majority of participants belong to the age group 21–30 years old as more than (27 %), followed by the age group 31– 44 years old (23.08%), age group of 41 – 50 years old participated as (16.92%) while the age groups 10 – 20 and 51 – 60 years old participated as (13.85%) and the age group of 61 – 70 years old participated as (4.61%) (Figure 1). The majority of patients enrolled in the study were females as 36 (55.6%) while males participated as 29 (45.4%) only.

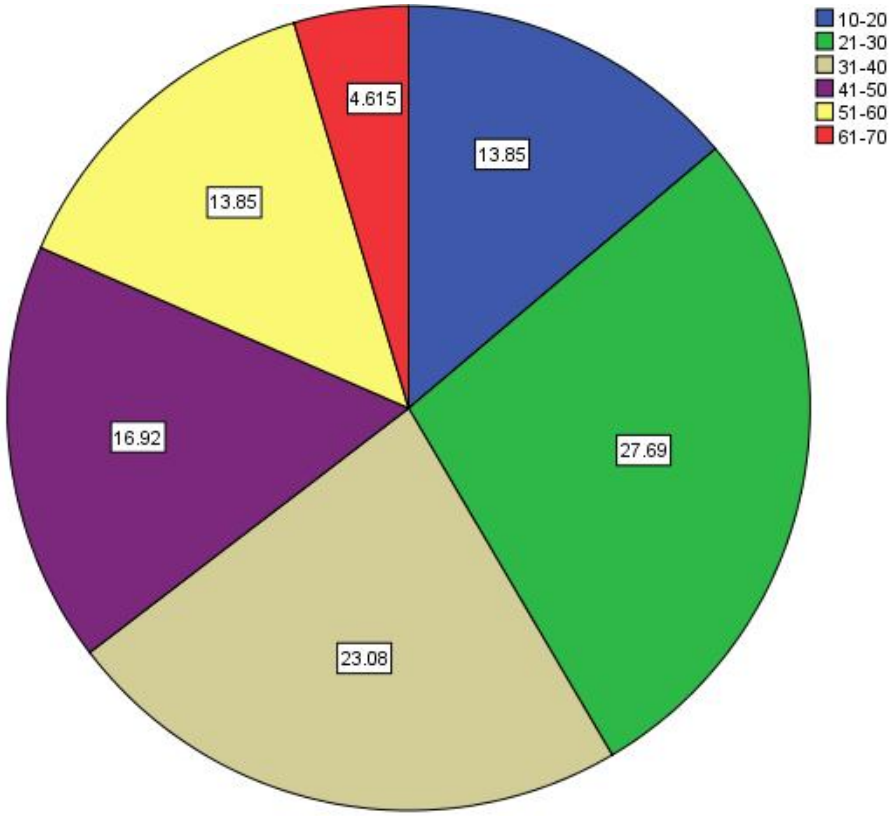

Fig 1: Age wise distribution of participants in the study.

Table 2: Type of samples collected in the study.

Sample	Frequency	Percent
Urine	25	38.5
Wound	30	46.2
Blood	10	15.4
Total	65	100.0

4.1. *Staphylocoocus aureus* antimicrobial Susceptibility testing

Thirty six (55.4%) of the isolates were sensitive to Methicillin, 25(38.5%) to Vancomycin, 27(41.5%) to Gentamicin and 29(44.6%) to Ciprofloxacin (Table 3).

Table 3: Sensitivity profile for all antibiotics used.

Antibiotic	Result	Count	%
Ciprofloxacin	Sensitive	29	44.6
	Resistance	36	55.4
Gentamicin	Sensitive	27	41.5
	Resistance	38	58.5
Vancomycin	Sensitive	25	38.5
	Resistance	40	61.5
Methicilin	Sensitive	36	55.4
	Resistance	29	44.6

4.2. Detection of virulent genes

The *Ica*was found predominating as the gene was detected in 48 (73.85%) of the isolates (Figure 2). Nineteen (29.2%) were found positive for *Cna* gene (Figure 3). Five (7.6%) isolates were found positive for *Hlg* gene (Figure4). *SdrE* as 25 (38.46%) were found positive for the presence of the gene (Figure 5). Cross positivity of different genes among isolates are summarized in (Table 4).

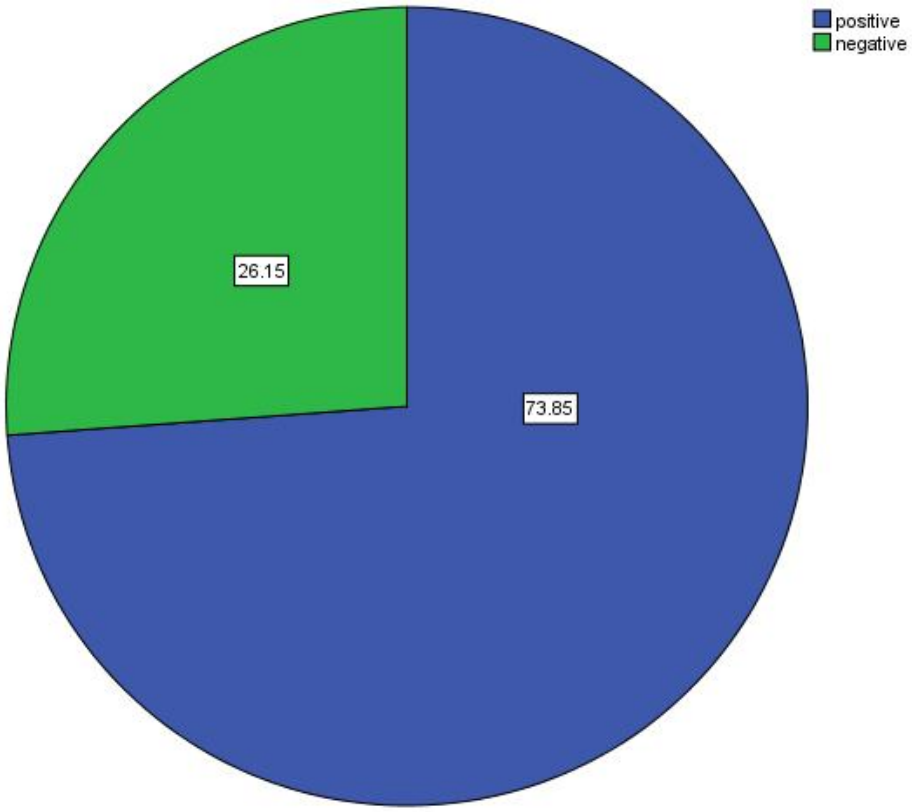

Fig 2: Frequency of *IcaS. aureus*gene among isolates.

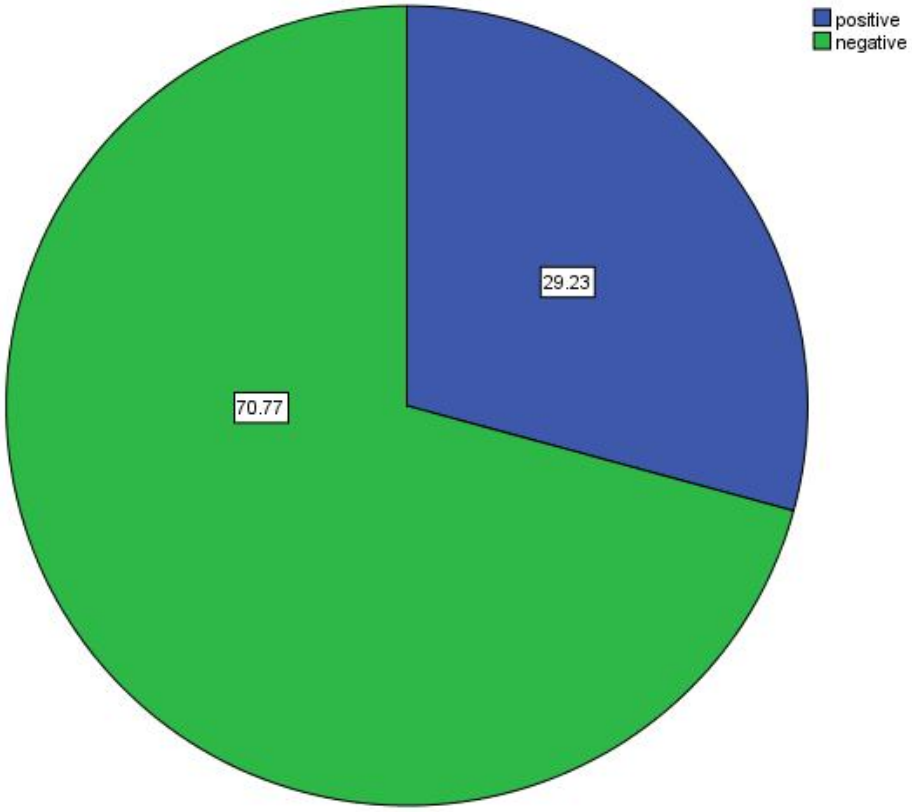

Fig 3: Frequency of *CnaS. aureus*gene among isolates.

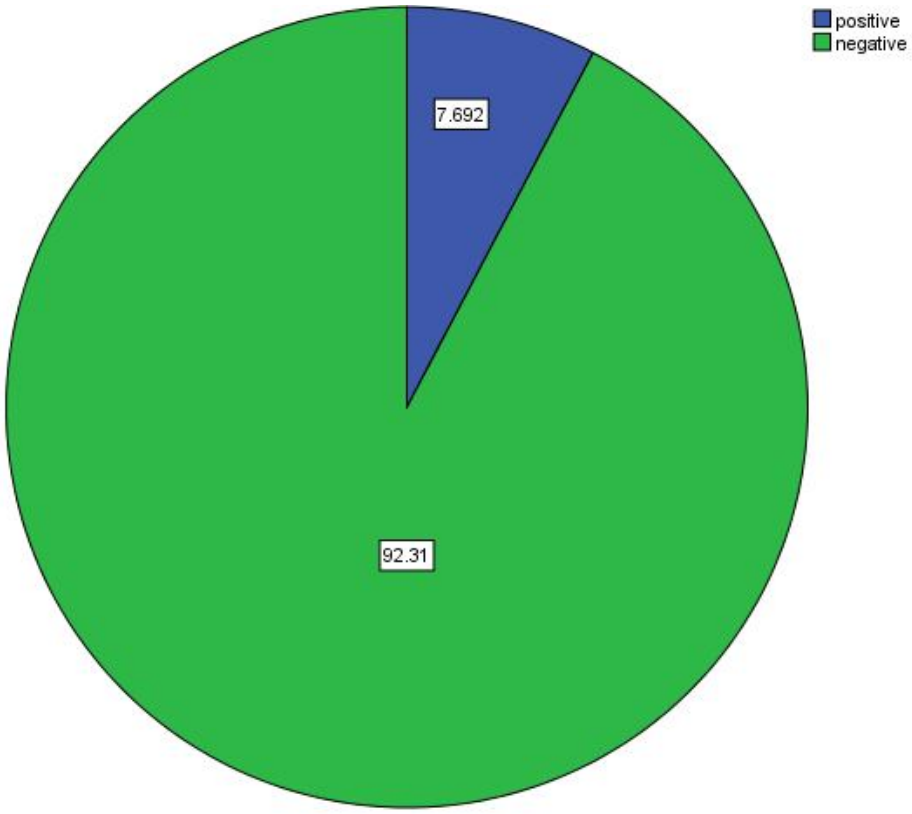

Fig 4: Frequency of *HlgS. aureus*gene among isolates.

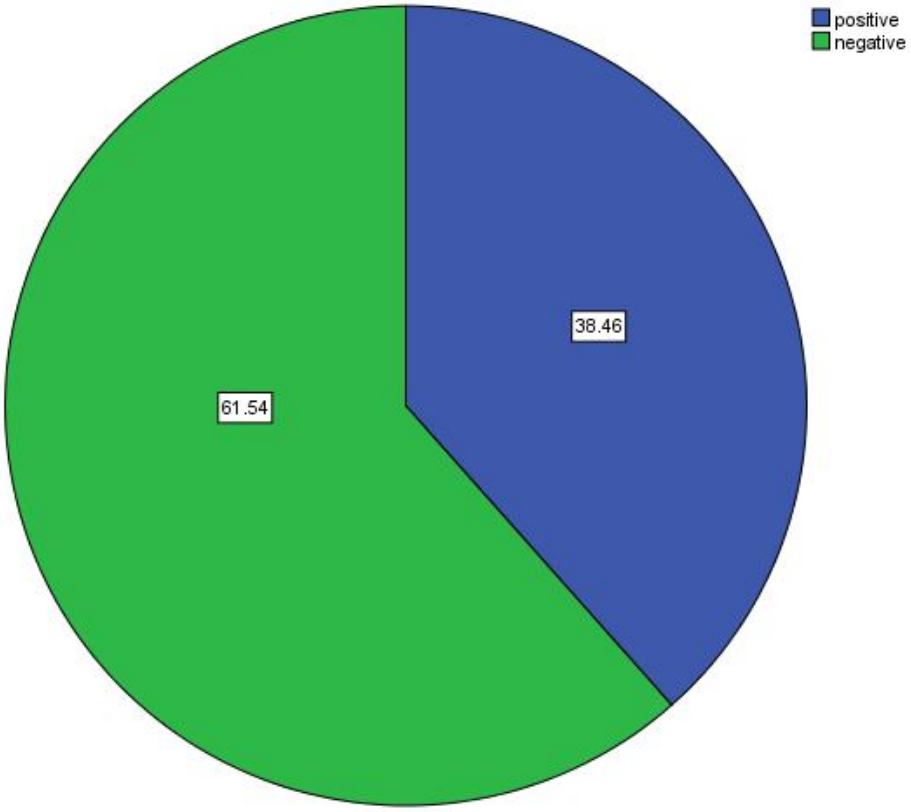

Fig5: Frequency of *SdrES. aureus*gene among isolates.

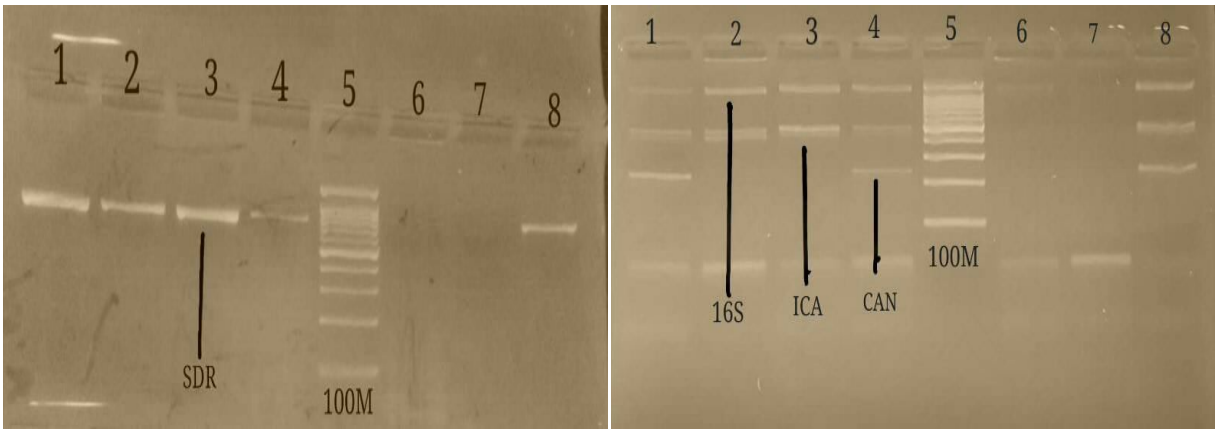

Fig 6: Detection of virulent genes by gel electrophoresis.**On the right**:**1 and 2 and 3 and 4:***Ica* and *Cna*positive, **5:** DNA ladder, **6 and 7:** Negative, **8:***Ica* and *Cna* positive. **On the left**:**1 and 2 and 3 and 4:***SdrE*positive.**5:** DNA ladder, **6 and 7:** Negative, **8:***SdrE* positive.

Table 4: Cross positivity of different *S. aureus*genes.

Gene	Reaction	*Cna*		*Ica*		*Hlg*		*SdrE*	
		positive	negative	Positive	negative	Positive	negative	positive	negative
		Count	Count	Count	Count	Count	Count	Count	Count
Cna	Positive	19	0	19	0	1	18	9	10
	Negative	0	46	29	17	4	42	16	30
Ica	Positive	19	29	48	0	4	44	19	29
	Negative	0	17	0	17	1	16	6	11
Hlg	Positive	1	4	4	1	5	0	2	3
	Negative	18	42	44	16	0	60	23	37
SdrE	Positive	9	16	19	6	2	23	25	0
	Negative	10	30	29	11	3	37	0	40

The relationship between virulent genes and antibiotic resistance as summarized in (Table 5) was indicated significant relationships (p=.03) between Ciprofloxacin resistance and the presence of *SdrE* gene as well as between Methicillin resistance and the presence of *SdrE* and *Ica* genes (p= .00 for both). No other significant associations were detected.

Table 5: The relationship between *S. aureus* virulent genes and antibiotic resistance.

Gene	Reacation	Ciprofloxacin Association		Gentamicin Association		Vancomycin Association		Methicilin Association	
		Resistance	*P**	Resistance	*P**	Resistance	*P**	Resistance	*P**
Cna	positive	9	.95	11	.32	13	.94	7	.11
	Negative	27		27		27		22	
Ica	positive	25	.69	28	.95	31	.21	20	.00
	Negative	11		10		9		9	
Hlg	positive	2	.31	4	.32	4	.31	2	.12
	Negative	34		34		36		27	
SdrE	positive	18	.03	15	.84	17	.94	17	.00
	Negative	18		23		23		12	

**P* value of significance at confidence level 95%.

Distribution of virulent genes according to gender and age as illustrated in (Tables 6&7) indicate no significant associations in any *Staphylocoocusaureus* virulent genes in the current study with age or gender. Regarding type of samples as summarized in (Table 8), significant association (p= .00) has been detected between *Hlg* gene and the type of sample, and no other significant relationships were detected.

Table 6: Relationship between virulent *S. aureus*genes and gender.

Gene	Reaction	sex				P value
		Males		Females		
		Count	%	Count	%	
Cna	positive	8	42.1	11	57.9	.79
	negative	21	45.7	25	54.3	
Ica	positive	22	45.8	26	54.2	.74
	negative	7	41.2	10	58.8	
Hlg	positive	1	20.0	4	80.0	.24
	negative	28	46.7	32	53.3	
SdrE	positive	11	44.0	14	56.0	.94
	negative	18	45.0	22	55.0	

Table 7: Relationship between virulent *S. aureus*genes and age groups.

Gene	Reaction	Age groups											
		10-20		21-30		31-40		41-50		51-60		61-70	
		Count	%	Count	%	Count	%	Count	%	Count	%	Count	%
Cna	positive	4	21.1	3	15.8	4	21.1	4	21.1	3	15.8	1	5.3
	negative	5	10.9	15	32.6	11	23.9	7	15.2	6	13.0	2	4.3
Ica	positive	6	12.5	11	22.9	11	22.9	10	20.8	7	14.6	3	6.3
	negative	3	17.6	7	41.2	4	23.5	1	5.9	2	11.8	0	0.0
Hlg	positive	1	20.0	0	0.0	0	0.0	2	40.0	2	40.0	0	0.0
	negative	8	13.3	18	30.0	15	25.0	9	15.0	7	11.7	3	5.0
SdrE	positive	3	12.0	7	28.0	6	24.0	4	16.0	3	12.0	2	8.0
	negative	6	15.0	11	27.5	9	22.5	7	17.5	6	15.0	1	2.5

Table 8: Relationship between virulent *S. aureus*genes and type of sample.

Gene	Reaction	Sample						P value
		Urine (25)		Wound swap (30)		Blood (10)		
		Count	%	Count	%	Count	%	
Cna	Positive	7	36.8	8	42.1	4	21.1	.71
	Negative	18	39.1	22	47.8	6	13.0	
Ica	Positive	19	39.6	21	43.8	8	16.7	.78
	Negative	6	35.3	9	52.9	2	11.8	
Hlg	Positive	0	0.0	1	20.0	4	80.0	.00
	Negative	25	41.7	29	48.3	6	10.0	
SdrE	Positive	9	36.0	13	52.0	3	12.0	.71
	Negative	16	40.0	17	42.5	7	17.5	

Chapter Five

Discussion, Conclusion and Recommendations

Chapter Five

Discussion, Conclusion and Recommendation

5.1. Discussion

In this study the prevalence of some *S. aureus*virulence markers in various clinical sampling was determined. A total of 65*S. aureus* after performing various biochemical assays was isolated.

Though several virulence-associated genes in *S. aureus* are known, the present preliminary study focused only on a small subset. These (*cna, hlg, ica* and*SdrE*) were chosen because they have been determined to be more common among invasive isolates. Amplification of the genes revealed that all four were present among the different *S. aureus* isolates in the current study. The number of different combinations found, in which all four selected genes were present in only four isolates, indicates high level of genetic diversity among the study population. Stotts and colleagues concluded that eight isolates from their study population (n =258) have these four genes in combination. In the present study, significant differences in antimicrobial susceptibility were observed between those *S. aureus* isolates carrying all four virulence genes and those carrying variable or no virulence genes. The *S. aureus* isolates acquisition of virulence genes was found to be positively correlated to the isolates to be more sensitive. The reason for this may be the adjacent location of the resistance gene to the virulence gene (Stotts *et al.*, 2004; Campbell*et al.*, 2006). However, it is observed in the study isolates that the presence of *Ica* gene is significantly contributing to the resistance against Vancomycin and Methicillin (p= 0.04).

Lara *et al.*, 2013in their study concluded no prevalence of either*Cna*or*Ica* genes among *S. aureus* isolates. Moreover,thepresence of *Cna* gene, which encodes the collagen binding protein, is not consistent with the report of

Smeltzer and his colleagues that *S. aureus* isolates among their study do not possess this gene (Smeltzer *et al.,* 1997).

Regarding*Hlg* (gamma-hemolysin) genewhich was present in (7.69%) among the current study isolates, has been reported by Lara and colleagues in their study in Brazil to be absent in their *S. aureus* isolates(Lara *et al*., 2013)**.**

SdrE gene has been found to be (38.46%) prevalent among study isolates. The *SdrE* proteins in*S. aureus*are members of the (MSCRAMMs) family Microbial SurfaceComponents-Recognizing Adhesive Matrix Molecules that are encoded by the tandemly arrayed *SdrC,SdrD*and*SdrE*genes (Josephson *et al*., 1998). Although the precise role of *SdrE* adhesins in staphylococcal infection is not known, a strongcorrelation between the *SdrE*genes of *S. aureus* and certain diseases has been reported. Several studies reported a significantlyincreased prevalence of *SdrE*genes in invasive *S. aureus*strains, in *S. aureus*strains responsible for osteomyelitis, and in *S. aureus*isolates responsible for bone infections (Peacock *et al*., 2002; Trad*et al*., 2004).
In this work, regarding type of samples significant association (p= .00) has been detected between the presence of*Hlg* gene and the type of sample. Regarding age and gender; no significant correlations were detected in any of the virulent genes under the study. No literature – to our knowledge has been found regarding study of the association between the virulent genes studied in the current study and age gender. However, (Diawara., *et al*., 2014) in their study *Staphylococcus aureus* nasal carriage in hemodialysis centers of Fez, Morocco reported a significant association between younger age described in their study as from 15 - 30 years old and the carriage of *Staphylococcus aureus.*

5.2. Conclusion

The presence of high number of *Staphylococcus aureus* isolates with different levels of virulence determinant genes in Sudanese hospitals. The results also provide evidence that the presence of antimicrobial resistant strains of*Staphylococcusaureus*to the antibiotics: methicillin and vancomycin become remarkably widespread in Sudanese population.

5.3. Recommendations

1- Application of this study on larger study population to better determine the prevalence of the different virulence determinants in *Staphylococcus aureus* isolates in Sudan.

2- Application of theseapproaches on other microorganisms especially other Gram positive bacteria, since the DNA extraction from such cells being no more difficult.

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APPENDIX

Appendix 1

Questionnaire

Sudan University of Science and Technology

College of Graduate studies

Molecular Detection of *Staphylococcus aureus* Virulence Genes Isolated from Different Clinical Samples in Khartoum State

By: Maab Mohammed Edries Elboshra

Supervised by: Prof. YousifFadlallahHamedelnil

Name…….. Date:……

Index number: ……

Age:…… Gender:……

Type of samples……

Symptoms:

1-fever …….. 2- back pain……

3-burning sensation ……. 4- headache……..

5- vomiting……

Any treatment received……..

Culture result ……..

Sensitivity result……..

Appendix 2

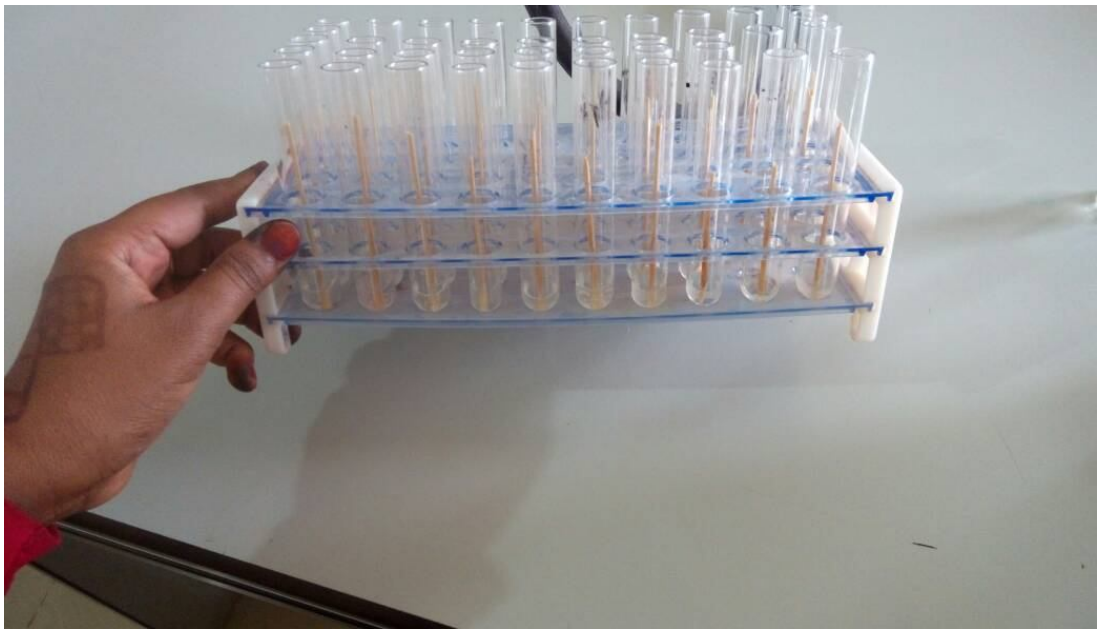

Fig 7: Catalase test.

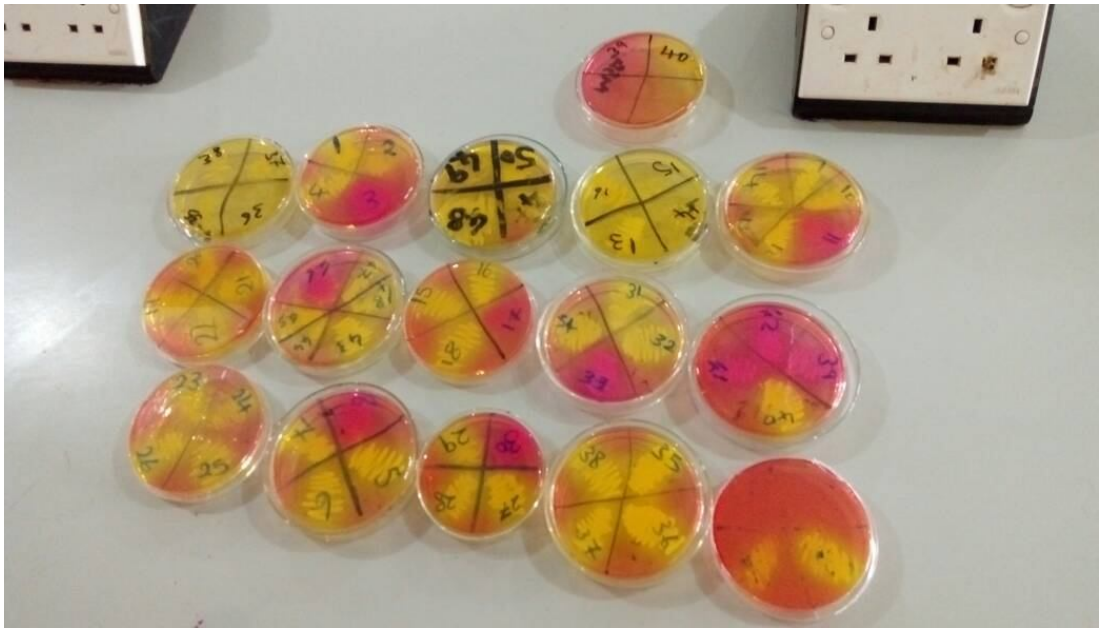

Fig 8: Growth on Manitol Salt Agar.

Fig 9:DNAes test.

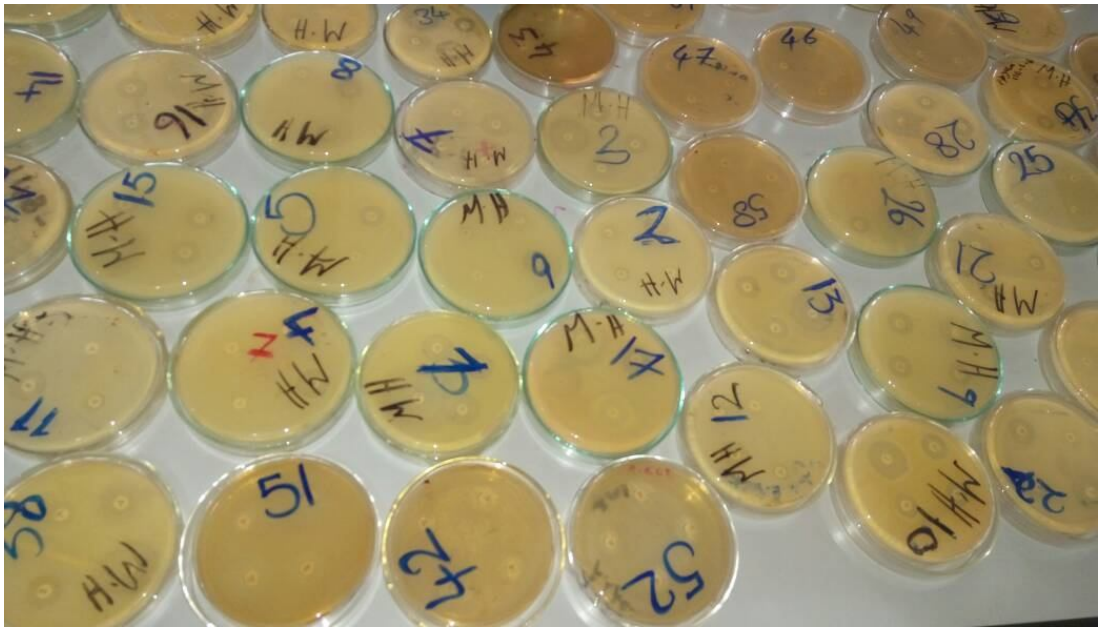

Fig 10: Antimicrobial susceptibility tests.

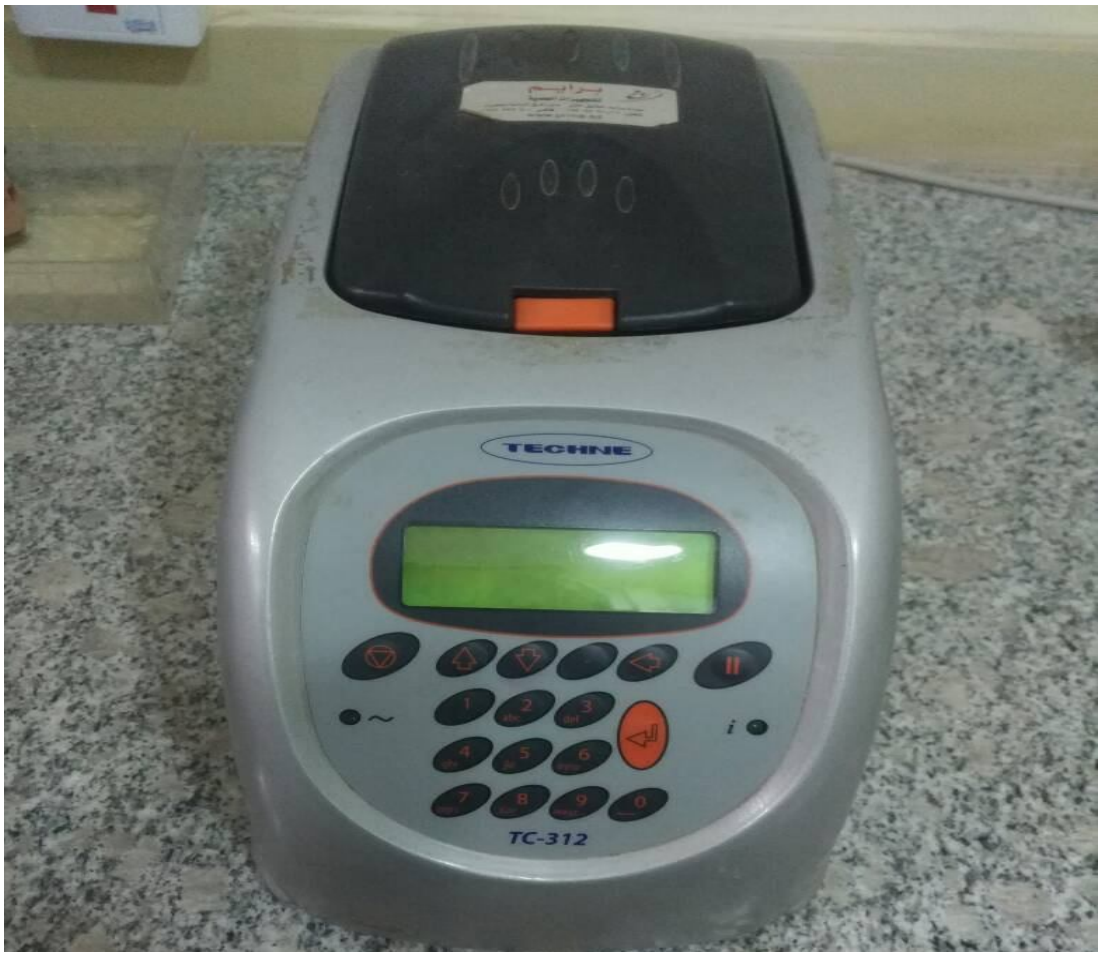

Fig 11:PCR machine.

Fig 12: Microcentrefuge used in the study.

Fig13: Gel electrophoresis.

Appendix 3

Reagents and media

Gram Stain (Cheesebrough, 2000)

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

Requirements

Crystal violet Gram stain (Hi Media)

To make 1 liter:

Crystal violet…… ... ... …20 g

Ammonium oxalate…… .....……9 g

Ethanol or methanol, absolute…… .....… ....….95 g

Distilled water…… to 1 liter

Lugol's iodine (Hi Media)

To make 1 liter:

Potassium iodide…… .....……20 g

Iodine……..10 g

Distilled water…….. To 10 liter

70% alcohol

Absolute alcohol…… .....…..70 ml

Distilled water…… .....…….30 ml

Saffranin (HiMedia)

Method of Preparation

• The dried smear was fixed by heat.

• The fixed smear was covered with crystal violet for 30-60 minutes.

• The stain was washed off with clean water.

• All water was tipped and the smear covered with lugol‟s iodine for 30-60 minutes.

• The stain was washed off with clean water.

• 70% alcohol was rapidly applied for 10-20 seconds for decolourization and then washed rapidly with clean water.

• The smear then covered with Saffranin stain for 2 minutes.

• The stain was washed off with clean water, back of slide was cleaned.

• After air-dry, smear was examined microscopically by using X 100 lens.

Results

Staphylococcus aureus appear as Gram posative cocci.

Preparation of Turbidity Standard

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

- 1.17% w/v solution of barium chloride was prepared by dissolving of2.35g of dehydrated barium chloride (BaCl2.2H2O) in 200 ml of distilled water.
- 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.
- A small volume of the turbid solution was transferred to screw-caped bottle of the same types as used for preparing the test and control inoculate (Chemie, 2014).

Coagulase test (Cheesebrough, 2000).

Principle**:** The free coagulase secreted by *S. aureus* reacts with coagulase reacting factor(CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrinresulting in clotting of plasma.

Procedure**:** 0.5 ml of oxalated or citrated plasma were added to a tube and mixed incolonies from a plate or add 0.5 ml of broth culture to the tube. The tube was covered toprevent evaporation and incubated at 37^{o}c. The tests were read by slowly tilting thetube. A positive test results in a highly viscous clot formation in the plasma. Once acoagulum, no matter how small, has formed the test is considered positive (usually within4 hours). A negative test results in the plasma remaining free flowing with no evidence ofa clot, were incubated overnight before a test is called negative, but prolonged incubation(over 24 hours) may result in the dissolution of a formed clot.

Catalase test(Cheesebrough, 2000).

Principle**:** the breakdown of hydrogene pereoxide in to oxygen and water is medieted by the enzyme catalase.

Procedure**:** a loop of bacterial growth is taken from nutrient agar medium.then the bacterialcell placed on a clean microscopic slide and a drop of 3% hydrogen peroxide is added. Aneffectiveness of oxygen gas, within a few seconds, indicates a positive reaction.

Manitol salt agar(oxoid, England)

Composition (g/l): lab-lemco poweder 1.0; peptone 10.0; mannitol 10.0: sodium chloride75.0; phenol red 0.025; agar 15.0 PH: 7.5± 0.

Direction: suspend 111 g in 1 liter of distilled water. Bring to the boil to dissolve completely, sterilize by autoclaving at 121^{o}c for 15 minutes.

Procedure**:** All the colonies that were collected through the necessary identificationtests (catalase and coagulase test) were streaked on manitol salt agar which is selectivemedia for members of Staphylococci and the bacterium were incubated at 37^{o}c for about24 hr. A positive result showing growth and a clear media change from red to yellow.

Antibiotic sensitivity testing

Inoculums Preparation

At least three to five well-isolated colonies of the same morphological type were selectedfrom an agar plate culture. The top of each colony was touched with a loop, and the growthswere transferred into a tube containing 4 to 5 ml of a

suitable broth medium, such as trypticsoy broth.The broth culture was incubated at 35-37^{o}C until it achieves or exceeds theturbidity of the 0.5 McFarland standards (usually 2 to 6 hours). The turbidity of the activelygrowing broth culture was adjusted with sterile saline or broth to obtain turbidity opticallycomparable to that of the 0.5 McFarland standards. This standard is prepared by adding 0.5 mlof 1% (11.75g/liter) $Bacl_2 2H_2O$to 99.5 ml of 1% (0.36N) H_2SO_4.

Inoculation of Test Plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculums suspension, asterile cotton swab was dipped into the adjusted suspension. The swab were rotated severaltimes and pressed firmly on the inside wall of the tube above the fluid level. This will removeexcess inoculums from the swab. The dried surface of a Mueller-Hinton agar plate wasinoculated by streaking the swab over the entire sterile agar surface. This procedure isrepeated by streaking two more times, rotating the plate approximately 60^{o} each time to ensure an even distribution of inoculums. As a final step, the rim is left 3 to 5 minutes, but nomore than 15 minutes, to allow for any excess surface moisture to be absorbed beforeapplying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs was dispensed onto the surface of theinoculated agar plate. Each disc was pressed down to ensure complete contact with the agarsurface. Whether the discs are placed individually or with a dispensing apparatus, they mustbe distributed evenly so that they are no closer

than 24 mm from center to center. Ordinarily,no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100mm plate. Because some of the drug diffuses almost instantaneously, a disc should not berelocated once it has come into contact with the agar surface. Instead, place a new disc inanother location on the agar. The plates were inverted and placed in an incubator set to 35^{o}Cwithin 15 minutes after the discs are applied.

Reading Plates and Interpreting Results

After 16 to 24 hours of incubation, each plate was examined. Zones were measured to thenearest whole millimeter, using sliding calipers or a ruler, which was held on the back of theinverted Petri plate. The petri plate were held a few inches above a black, nonreflectingbackground and illuminated with reflected light.