Sudan University of Science and Technology

College of Graduate studies

Molecular Detection of Staphylococcus aureus Enterotoxin A to E from Different Clinical Sample in Khartoum State

التعرف الجزيئي للجينات المسببة للسموم في باكتيريا المكورات العنقودية الذهبية من عينات سريرية في ولاية الخرطوم

A dissertation Submitted in Partial Fulfillment of M.Sc. degree of Medical Microbiology

By:

Lemya Hassan Mahmoud

Faculty of Medical Laboratory Science

Alzaiem Alazhari University

2000

Supervisor: Prof. yousif Fadlallah Hamedelnil

2017

Sudan University of Science & Technology

College of Graduate Studies

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

كلية الدراسات العليا

Ref: SUST/ CGS/A11

Approval Page

(To be completed after the college council approval)

Name of Candidate: Lemya Hassan Mahmoud Abdelatif

Thesistitle: Molecular Detection of Staphylococcus aureus toxin genes Isolated from different Clinical samples in Khartoum State

Degree Examined for: Ph.D

Approved by:

1. External Examiner

Name: Abbas Mohmed Ahmed

Signature: .... Date: 28/8/2017

2. Internal Examiner

Name: Hisham Nouraldayem Altayeb Mahammed

Signature: .... Date: 28/8/2017

3. Supervisor

Name: Yousif Fadlalla Hamedelnil

Signature: .... Date: 6-9-2017

الآيـة

{وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ}

آية رقم (105) من سورة التوبة

DEDICATION

The research work is dedicated to the beloved ones; my father, my mother, my husband and my children

ACKNOWLEDGEMENT

Thanks the Almighty ALLAH for making it possible for me to complete this work successfully. My heartfelt gratitude goes to my project supervisor Prof. Yousif Fadlalla for his unlimited support and guidance.

ABSTRACT

The aim of this study was to detection of *Staphylococcus aureus* toxin genes isolated from different clinical samples in Khartoum state by multiplex PCR assay. A total of 80 clinical specimens (30 urine, 30 wound swab and 20 blood) were collected in this study. Urine samples were culture on CLED agar, while wound swab and blood samples were culture on blood agar and MacConky agar, identification was done by catalase test, coagulase test, DNAse test and culture on manitol salt agar. Molecular confirmation was done by 16s housekeeping gene, 65 samples are found positive for 16s, while 15 samples are negative for 16s and this samples was excluded from study. Modified Kirby-bauer method was performed using the following antibiotic discs; Gentamicin, Vancomycim,, Ciprofloxacin and Methicilin. Fifty five percent of samples were found sensitive to Methicilin, 42% were sensitive to Gentamicin, 45% were sensitive to Ciprofloxacin and 38% were sensitive to Vancomycim. Guanidine hydrochloride method was adopted for DNA extraction. Finally Multiplex PCR was done for the detection of *Staphylococcus aureus* toxin genes (*SA-A, SA-B, ENT-C, SA-D and SA-E*). Most study population were females 36(55%); 19 of them suffering from UTIs, 12 suffering from wound inflation and 5 of them suffering from bacteremia, while males were 29(45%); 6 of them were suffering from UTIs, 18 of them were suffering from wound infection and 5 of them suffering from bacteremia. Among enrolled subjects, 56 were positive for one or more *Staphylococcus aureus* toxin genes. While 9 isolates were negative for all toxin genes. The results of multiplex PCR: five (n=5) blood samples appear as *SA-A* toxin gene positive and this gene was not detected in urine and wound swab samples. Nine (n=9) urine samples appear as *SA-B* toxin gene positive, twelve (n=12) wound swab samples appear as positive and four (n=4)

blood samples appear as positive. Eleven (n=11) urine samples appear as *ENT-C* toxin gene positive, nine (n=9) wound swab samples appear as positive and two (n=2) blood samples appear as positive. one (n=1) urine samples appear as *SA-E* toxin gene positive, three (n=3) wound swab samples appear as positive and this gene was not detected in blood samples. *SA-D* toxin gene was not detected in any sample. The study concluded that *SA-B* was found predominant, and strong association (P-value 0.000) between *SA-A* toxin gene and blood samples.

المستخلص

هدفت هذه الدراسة الى تحديد الجينات المسؤولة عن افراز عدد من السموم في بكتريا المكورات العنقودية الذهبية المعزولة من عينات سريرية في ولاية الخرطوم, حيث تم الكشف عنها عن طريق فحص تفاعل البلمرة المتعدد المحتوي علي عدة بادئات. تم جمع80 عينة (30 عينة بول، 30 عينة جروح ملتهبة, 20 عينة دم) في هذه الدراسة. ثم تم تزريع عينات البول علي وسط CLED وعينات الجروح الملتهبة و الدم في علي وسط blood agar و MacConkey و تم التعرف علي البكتريا باختبارcatalase, و اختبار coagulase, و اختبار DNAes, و تم تزريعها في وسط manitol salt agar. تم التاكيد بواسطة الاختبار الجزئي عن طريق جين 16s وجد ان 65 عينة كانت ايجابية للجين 16s, 15 عينة سالبة للجين 16s وتم استبعاد العينات السلبية من الدراسة. تم استخدام طريقة Kirby-bauer المطورة لاجراء إختبار الحساسية باستخدام اقراص المضادات الحيوية التالية: جنتاميسين، فانكومايسين، سيبروفلوكساسين وميثيثليين وجدنا أن 42% من العينات حساسة للجنتاميسين، 55٪ حساسة للميثيثليين، 45٪ حساسة للسيبروفلوكساسين و 38٪ حساسة فانكومايسين. تم استخدام طريقة hydrochloride Guanidine لاستخراج الحمض النووي. اخيرا تم إجراء إختبار تفاعل البلمرة المتعدد المحتوي علي عدة بادئات للكشف عن جينات السموم (*SA-A, SA-B, ENT-C, SA-D, SA-E*) في بكتريا المكورات العنقودية الذهبية.

وكان معظم المشاركين في الدراسة من الإناث 36 (55٪)؛ 19 منهم يعانون من عدوى المسالك البولية و 12 منهم يعانون من الجروح الملتهبة و 5 منهم يعانون من بكتريا الدم، في حين بلغ عدد الذكور 29 (45٪)؛ 6 منهم يعانون من التهاب المسالك البولية و 18 منهم يعانون من الجروح الملتهبة و 5 منهم يعانون من بكتريا الدم. من عينات الدراسة، كانت 56

عينة إيجابية لواحدة أو أكثر من جينات السموم . بينمـــا كانـــت 9 عينـــات ســـلبية لجميـــع الجينـــات. نتيجة اختبار تفاعـــل البلمـــرة المتعـــدد المحتـــوي علـــي عـــدة بادئـــات: خمســـة (ن=5) عينـــات دم ظهرت موجبة للجين *SA–A* و لم يظهر الجـــين فـــي عينـــات الجـــروح الملتهبـــة و الـــدم. تســـعة (ن=9) عينات بـــول ظهـــرت موجبـــة للجـــين *SA–B*, اثناعشـــر (ن=12) عينـــة جـــروح ملتهبـــة ظهرت ايضا موجبة, اربعـــة (ن=4) عينـــات دم ظهـــرت موجبـــة. احـــدي عشـــر (ن=11) عينـــات بول ظهرت موجبة للجين *ENT–C*, تسعة (ن=9) عينات جـــروح ملتهبـــة ظهـــرت ايضـــا موجبـــة, عينتين (ن=2) دم ظهرت موجبة. عينـــة بـــول واحـــدة (ن=1) ظهـــرت موجبـــة للجـــين *SA–E*, ثلاث (ن=3) عينات جروح ملتهبة ظهرت ايضـــا موجبـــة, و لـــم يظهـــر الجـــين فـــي اي عينـــة دم. الجين *SA–D* لم يظهر في اي عينـــة. وخلصـــت الدراســـة إلـــى أن الجـــين الســـموم *SA–B* كـــان الاكثر ظهورا في هذه الدراسة, و وجـــدت علاقـــة قويـــة (P–value 0.000) بـــين الجـــين *SA–A* و عينات الدم.

TABLE OF CONTENTS

CHAPTER ONE

الآية I
Dedication II
Acknowledgement III
Abstract IV
المستخلص VI
Table of contents VIII
List of tables and figures XI

1.1. INTRODUCTION

1.1.1. Background 2
1.1.2. Rationale 4
1.1.3. Study Objectives 5

CHAPTER TWO

2. LITERATURE REVIEW

2.1. *Staphylococcus* 6
2.2. *Staphylococcus aureus* 7
2.3. Virulence factors 8
2.4. Staphylococcal Enterotoxins 9
2.4.1. Staphylococcal Enterotoxin Properties 10
2.4.2. Staphylococcal Enterotoxin Mechanism of action 11
2.4.4. Staphylococcal Enterotoxin Nomenclature 11
2.5. Antibiotic Resistance in *S. aureus* Correlation to Enterotoxin production 11
2.6. Serological assays employed in the detection of staphylococcal 14

enterotoxins
2.6.1. Gel diffusion assay 14
2.6.2. Electrophoresis assay 14
2.6.3. immunofluorescent assay 15
2.6.4. hemagglutination assay 15
2.6.5. Enzyme immunoassay (EIA) and Enzyme linked immunosorbent assay (ELISA) 15
2.7. Detection of Enterotoxin genes 16
2.7.1. PCR 16
2.7.2. Microarrays 17

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design 18
3.2. Study area 18
3.3. Study population 18
3.4.1. Inclusion criteria 18
3.4.2. Exclusion criteria 18
3.5. Ethical Consideration 18
3.6. Data collection 19
3.7. Laboratory work 19
3.7.1. Specimen collection and Cultivation 19
3.7.2. Identification 19
3.7.3. In- Vitro antibiotic sensitivity testing 20
3.7.4. McFarland standard 20
3.8. DNA extraction 21
3.9. Detection of *Staphylococcus aureus* Enterotoxin by Multiplex PCR 22

3.10. Visualization of the DNA product 23
3.11. Statistical Analysis 24

CHAPTER FOUR

RESULTS

The association between the *S.aureus* Enterotoxin genes and Age group 25
The association between the *S.aureus* Enterotoxin genes and gender 26
The association between the *S.aureus* Enterotoxin genes and clinical samples 27
The association between the *S.aureus* Enterotoxin genes and Antibiotic 29

CHAPTER FIVE

DISCUSSION, CONCLUSION and RECOMMENDATIONS

DISCUSSION 33
CONCLUSION 36
RECOMMENDATIONS 37

REFERENCES 38
APPENDIX 49

LIST OF TABLES AND FIGURES

Title of Table/Figure	Page No
Details of primers used in the study and amplicon sizes	23
group of enrolled patients and relationship with Enterotoxin genes	25
The association between the *S.aureus* Enterotoxin genes and gender	26
The association between the *S.aureus* Enterotoxin genes and clinical samples	27
Agarose gel electrophoresis of multiplex PCR product	28
The association between the *S.aureus* Enterotoxin genes and Mithecillin	29
The association between the *S.aureus* Enterotoxin genes and Vancomycin	30
The association between the *S.aureus* Enterotoxin genes and Gentamicin	31
The association between the *S.aureus* Enterotoxin genes and Ciprofloxacin	32

Chapter One

INTRODUCTION

1.1. Background

Staphylococcus aureus is a Gram-positive bacterium produces enterotoxins and virulence factors, which are causative agents of foodborne intoxications, and other infections. Staphylococcal enterotoxins (SE) are a large family of nine serotypes of heat stabile toxins that cause gastroenteritis and are potent superantigens that cause T-cell stimulation (Balaban and Rasooly, 2000). The most important of these is enterotoxin A (SEA) which is the most common SE associated with food-borne illness in humans. The mode of action is not clearly understood but it is thought to be mediated via vagal stimulation on the emesis centre of the brain and activation of local and systemic immune responses. (Argudin *et al*., 2010). They are classified into classical and novel enterotoxins. Enterotoxins are single chain polypeptides and have a molecular weight of about 26-28 kDa and 228-239 amino acid residues the organism is a commensal and lives on the anatomical locales of humans and animals. Sensitive detection methodologies for enterotoxins are important because enterotoxins are potent superantigens and could possibly be used as a biological warfare weapons (Muller-Alouf *et al.,* 2001). Staphylococcal enterotoxins are also commonly found in mastitis isolates from cows, although there is geographic variation in isolates with regards to prevalence and types and their role in pathogenesis is poorly understood (Larsen *et al*., 2002). Staphylococcus enterotoxin C (SEC) has been shown to activate bovine neutrophils in vitro (Ferens *et al*., 1998) and cows infected with Staphylococcus enterotoxin D (SED)-producing strains secrete SED into milk and induce SED-specific humoral and cell-mediated immune responses (Tollersrud *et al*., 2006).

The characterization of the enterotoxin genes and the enterotoxigenic isolates of *S. aureus* can provide researchers with additional information in an event of an outbreak.

Many methods have been developed for detection of the presence of enterotoxins in foods. These methods are based on the presence of detectable amounts of toxins. The methods are immunoassays, as the bioassays were unreliable, cumbersome and expensive. Recently, many methodologies have been developed using biosensor technology. The methods employing signal amplifiying technology can also greatly improve the sensitivity of detection of toxins. The conjugation of antibodies to oligonucleotides has led to signal amplification technologies in which, specificity of antigen antibody interactions and massive amplification potential of polymerase chain reaction (PCR) could be put to use to obtain sensitive detection of enterotoxins (Muller-Alouf *et al*., 2001).

The enterotoxin genes of *S. aureus* are present on regions known as the staphylococcal pathogenicity islands (SaPIs). The genes could also be plasmids. Many isolates of *S. aureus* harbor multiple enterotoxin genes. Characterization of enterotoxigenic isolates and enterotoxin genes is necessary to develop in depth knowledge about these toxic agents. Enterotoxin genes have been identified by methods like DNA hybridizations and polymerase chain reactions. These methods are reliable alternatives to other cumbersome and expensive methods like microarrays. PCR based methods like multiplex PCR has provided a rapid means to identify the presence or absence of desired genes. However, methods developed should be able to provide more information on enterotoxin genes, so that genes and isolates could be characterized. Methods involving sequencing of genes and methods like single/multi locus toxin sequence typing can provide more information on genes and the enterotoxigenic isolates. 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; Muller-Alouf *et al*., 2001).

1. 2. Rationale

Staphylococcus aureus causes a wide range of infections that may be broadly divided into community and hospital acquired. Community-acquired infections include toxin-mediated diseases such as TSS and food poisoning, infections affecting the skin and soft tissues, infection of bones and joints, infection relating to other deep sites (e.g. endocarditis, abscess formation in liver and spleen) and infection of lung and urinary tract. This wide range of manifestations occurs both in predisposed and in previously healthy individuals. The commonest deep-site infections are endocarditis and bone and joint infection (Fowler *et al*. 2003).

Staphylococcal food poisoning results from ingestion of preformed SEs (Dinges, *et al,* 2000). This occurs when *S. aureus* is inoculated into food which is then left in conditions that are permissive for bacterial multiplication and toxin secretion before consumption. The mechanism of action of the toxin is still under study, but the initiation of the emetic response is thought to be due to interactions with the emetic reflex located in the abdominal viscera, with subsequent activation of the medullary emetic centre in the brain stem that is stimulated via the vagus and sympathetic nerves (Dinges, *et al*, 2000). Nausea and vomiting occur after an incubation period of 2 and 6 h. Abdominal pain and diarrhoea are also common features. Diagnosis is made on clinical grounds. Suspected food may be cultured for the presence of *S. aureus*.

Sensitive detection methodologies for enterotoxins are important because enterotoxins are potent superantigens and could possibly be used as a biological warfare weapons. Detection of enterotoxigenic strains of *S. aureus* is also important for epidemiological reasons.

1. 3. Study Objectives

1. 3.1. General Objective

To detection of *Staphylococcus aureus* Enterotoxin A to E from Different Clinical Samples in Khartoum State

1. 3.2. Specific Objectives

1. To isolate *S. aureus* from different clinical samples

2. To screen the isolated *S. aureus* for the antimicrobial susceptibility testing to most commonly used antibiotics.

3. To characterize the toxins genes of *S. aureus* (*SA-A, SA-B, ENT-C, SA-D and SA-E*) by multiplex PCR.

4. To compare between the presences of *S. aureus* toxins genes in different clinical samples.

5. To compare the presence *S. aureus* toxins genes with gender, age group and antimicrobial susceptibility testing.

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, 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).

The first reports on staphylococci date from 1880, when Alexander Ogston reported upon the isolation of *S. aureus* from a surgical wound infection (Ogston, 1880). The organisms were able to produce an abscess when injected into guinea pigs and mice (Ogston, 1880). Also in 1880, Louis Pasteur produced abscesses in animals injected with pus from human staphylococcal infections (Pasteur, 1880). In the following year, Ogston coined the term *Staphylococcus* for the genus (Ogston, 1882). In 1884, Rosenbach divided the genus into the species *aureus* and *albus*. These designations remained until 1939, when Cowan differentiated *S. epidermidis* as a separate species based upon coagulase testing (slide agglutination), and this was further defined with serological testing in 1964 (Cowan, 1964)

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* 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 (Vos *et al*., 2009; 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, 2009a).

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. 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). *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 first bacterial two-component toxin described and the first staphylococcal toxin identified was the Panton–Valentine leucocidin (PVL) (Panton *et al.*, 1932). α-Haemolysin (Hla) was selected because of its very long history of study and because a specific receptor has been identified. Toxic shock syndrome toxin-1 (TSST-1) is given as an example of a superantigen toxin. Protein A (SpA) is reviewed as an example of anti-host defence factor (Spaan *et al.*, 2013) for reviews on the many other factors produced by *S. aureus* to neutralize the host immune responses). Fibronectin binding protein (Fnb) is provided as an example of host tissue adhesin. Polysaccharide intercellular adhesin (PIA) is an example of a polysaccharide for accumulation in biofilm and anti-phagocytic factor. other virulence factors of *Staphylococcus* genus include surface components, such as the capsule, peptidoglycans, teichoic acid, protein A, collagen cell attachment protein, 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 (Spaan *et al.*, 2013).

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 (34) 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. Staphylococcal Enterotoxins

Staphylococcal enterotoxins are members of a family of more than 20 different staphylococcal and streptococcal exotoxins. These bacterial proteins are known to be pyrogenic and are connected to significant human diseases that include food poisoning and toxic shock syndrome. These toxins are for the most part produced by *S. aureus* although other species have also been shown to be enterotoxigenic. *S. aureus* is persistent in more than 20% of the general population, while another 60% are intermittent carriers, most frequently, the nose is the site of colonization in humans, and this colonization increases the risk of infections when host defenses are compromised. This is supported by multiple observations, for instance, the frequency of infections is higher in carriers than in non-carriers (von Eiff *et al*., 2001; Havelaar *et al*., 2010).

The chief function of these various enzymes, cytotoxins, exotoxins, and exfoliative toxins is to turn host components into nutrients that the bacteria may use for growth. Among the other secreted factors are exotoxins that include staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST)-1, these factors subvert the host immune system and illicit major responses (Morandi *et al*., 2009).

S. aureus enterotoxins (SEs) are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase (Derzelle *et al*., 2009). They are active in high microgram to low nanogram quantities (Larkin *et al*., 2009), and are resistant to

conditions (heat treatment, low pH) that easily destroy the bacteria that produce them (Argudin *et al*., 2010).

Most genes coding for SEs are located on mobile elements such as plasmids, bacteriophages or pathogenicity islands (Lindsay *et al*., 1998). Thus, horizontal transfer between strains is not rare, according to (Varshney *et al*., 2009), most *S. aureus* isolates obtained from three separate hospitals had more than one enterotoxin gene. Although there are more than 20 distinct staphylococcal enterotoxins, only a few of them have been studied in depth.

The most common staphylococcal enterotoxins are *SEA* and *SEB. SEA* is the most common toxin in staphylococcus-related food poisoning. *SEB* is associated as well with food poisoning, moreover, it has been studied for potential use as an inhaled bioweapon (Ler *et al*., 2006). SED is suggested to be the second most common staphylococcal toxin associated with food poisoning worldwide, and one study showed that only very small amounts of this toxin were needed to induce food poisoning. *SEE* has also been documented in some cases of food poisoning, while *SEF* has been implicated in toxic shock syndrome (Pinchuk *et al*., 2010). *SEG, SEH*, and *SEI* are not as well studied as the others, but they have been shown to be associated with one of the food poisoning outbreaks in Taiwan (Chen *et al*., 2004). *SEH* has been also identified as one of the causes of massive food poisoning associated with the reconstituted milk consumption in Osaka, Japan in 2000 (Ikeda *et al*., 2005).

2.4.1. Staphylococcal Enterotoxin Properties

These SE proteins have a remarkable ability to resist heat and acid, therefore, they may not be completely denatured by mild cooking of contaminated food. They are pyrogenic and share some other important properties that include the ability to induce emesis and gastroenteritis as well as their noted superantigenicity, they are resistant to inactivation by gastrointestinal proteases including pepsin, trypsin, rennin and papain, thus they can easily outlast the bacteria that produce them (Le Loir *et al*., 2003; Pinchuk *et al*., 2010).

2.4.2. Staphylococcal Enterotoxin Mechanism of action

Staphylococcal enterotoxins belong to the broad family of pyrogenic toxin superantigens (SAgs); SAgs bypass conventional antigen recognition by interaction with major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells, and with T-cell receptors (TCR) on specific T-cell subsets, interaction typically occurs to the variable region of the TCR β chain (Vβ) but binding to the TCR Vα domain has been reported, this leads to activation of a large number of T-cells followed by proliferation and massive release of chemokines and proinflammatory cytokines that may lead to potentially lethal toxic shock syndrome (Larkin *et al*., 2009).

2.4.4. Staphylococcal Enterotoxin Nomenclature

SEs have been proposed to be named according to their emetic activities, only SAgs that induce vomiting after oral administration in a primate model will be designated as SEs, related toxins that lack emetic activity or have not been tested for it should be designated as staphylococcal enterotoxin-like (SEls) SAgs, also, newly discovered toxins with more than 90% amino acid sequence identity with existing SEs or SEls should be designated as a numbered subtype, however, despite this consensus nomenclature some subtypes are still just called variants (Lina *et al*., 2004; Schlievert and Case, 2007).

2.5. Antibiotic Resistance in *S. aureus*

Resistance to β-Lactam Antibiotics

Penicillin-resistant *S. aureus* strains emerged in the early 1940s, shortly after the introduction of penicillin into clinical practice. Resistance to methicillin and other β-lactamase-resistant penicillins was likewise observed soon after methicillin was introduced into clinical use in Britain (Jevons, 1961). At this time the methicillinresistant strains isolated in Britain demonstrated heterogeneous resistance to

methicillin (i.e. affecting only a minority of the cell population), were multiply antibiotic resistant and were isolated from hospitalized patients (Barber 1961). After the mid-1970s, large outbreaks of infection caused by MRSA were reported in many hospitals in Britain (Shanson, *et al*. 1976; Cookson and Phillips 1988), Ireland (Cafferkey *et al*. 1985), the United States (Schaefler *et al*. 1981) and Australia (Pavillard *et al*. 1982). Many of these early MRSA epidemics were caused by a single epidemic strain that was transferred between hospitals (Duckworth *et al*. 1988). Since then, many clones of MRSA associated with epidemic spread or sporadic infections have been described throughout the world. Approximately one-third of serious *S. aureus* infections in the United Kingdom are now caused by MRSA, although the figure varies considerably worldwide. Until recently, MRSA was mostly confined to the hospital setting and MRSA colonization of those discharged from the community rarely persisted long term except when associated with defects in the skin integrity or the presence of prosthetic material. However, community-acquired MRSA associated with both colonization and infection is being increasingly recognized (Daum *et al*. 2002; Okuma *et al*. 2002). These strains are resistant to fewer non-β-lactam antibiotics than most of the previously defined MRSA strains.

Resistance to Vancomycin

Staphylococcus aureus with intermediate-level resistance to vancomycin (VISA) was first detected in Japan in 1996 (Hiramatsu *et al*. 1997b). The strain (Mu50) was isolated from an infant who developed a sternal wound infection that was refractory to treatment following surgery to correct a congenital cardiac defect. This strain was reported to have an MIC of 8mg/l (Hiramatsu *et al*. 1997b). VISA strains have since been identified worldwide, although they are currently rare in clinical practice, and most appear to evolve from MRSA strains in patients who have received prolonged vancomycin treatment. The first hVISA (Mu3) was identified in Japan from the sputum of a patient with MRSA pneumonia following surgery (Hiramatsu *et al*. 1997a). This strain was reported to have an MIC of 3 μg/ml (Hiramatsu *et al*. 1997a). Serial passage of Mu5 in

increasing concentrations of vancomycin gave rise to subpopulations with levels of resistance comparable to those of Mu50, and typing showed that Mu3 and Mu50 had the same PFGE pattern (Hiramatsu *et al.* 1997a). hVISA has since been reported from around the world and appears to be more common than VISA (Hiramatsu *et al.* 1997a; Hiramatsu 2001; Liu and Chambers 2003). The first VRSA was reported in Michigan in July 2002, with a second apparently unrelated case in Pennsylvania 2 months later (Centers for Disease Control and Prevention 2002a, 2002b).

Resistance to Other Antibiotics

Staphylococcus aureus plasmids have been classified into three general classes. Class I plasmids are small (1–5 kbp), have a high copy number (10–55 copies per cell) and are either cryptic or encode a single antibiotic resistance. These plasmids are the most widespread throughout the genus *Staphylococcus*. For example, the pT181 family comprises a group of small (4–4.6 kbp) plasmids that usually encode tetracycline or chloramphenicol resistance, and the pSN2 family plasmids often encode erythromycin resistance. Class II plasmids, commonly called penicillinase or β-lactamase plasmids, are relatively large (15–33 kbp), have a low copy number (4–6 per cell) and carry several combinations of antibiotic and heavy-metal resistance genes, many of which are located on transposons (Shalita, *et al.* 1980; Lyon and Skurray 1987). Class III plasmids appear to be assemblages of transposons and transposon remnants (Gillespie *et al.* 1987).

S. aureus strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier and because of their location within microabscesses, which limit the action of drugs (Gündogan *et al.*, 2006; Pesavento *et al.*, 2007).

It is well documented that determinants of resistance to antibiotics and other toxic substances in staphylococci, as in other pathogens, are largely carried by accessory genetic elements, especially plasmids, transposons and their relatives, a particularly

important resistance determinant in staphylococci is that for methicillin and other β -lactam compounds, giving rise to the notorious MRSA acronym. Although it has been clear for some time that a novel penicillin-binding protein, PBP2a, is responsible for this resistance, and that the PBP2a gene is not native to *S. aureus*, there has been considerable uncertainty regarding the nature of the accessory genetic elements that carry it. Several reports had analyzed the prevalence of enterotoxin genes in methicillin-resistant and methicillin-susceptible (MSSA) isolates of *S. aureus*, (Sila *et al*., 2009) found 7 genes more frequently detected in MRSA isolates: *sea, seb, sed, seg, sei, sej* and *eta*, coding for the production of enterotoxins A, B, D, G, I, J and the exfoliative toxin A. On the other hand, the *pvl, tst* and *sec* genes for Panton-Valentine leukocidin, TSST-1 and enterotoxin C were most frequent in MSSA.

2.6. Serological assays employed in the detection of staphylococcal enterotoxins

2.6.1. Gel diffusion assay

The enterotoxin antigen has to react with its corresponding antibody resulting in precipitation or agglutination reactions. In single or double diffusion assays the soluble antigen reacts with its specific antibody resulting in the formation of a visible precipitation line. Initially the assays were performed in tubes, where by the melted agar contained the antiserum. The agar was poured into (partially- filled) test tubes and was overlaid with solution containing the antigen, which is the enterotoxin. The interaction between antigen and antibody resulted in the formation of precipitin bands at the interface in the test tubes. In double diffusion, a layer of plain agar separated the antiserum containing molten agar and the enterotoxin solution. The antigen and the antibody migrate into the plain agar and form the precipitin bands (Casman and Bennett, 1964; Hall *et al*., 1965).

2.6.2. Electrophoresis assay

A quantitative electroimmunodiffusion method for detection of enterotoxin A was described by Gasper *et al*. (1973). The technique which was introduced by Laurell

(1966) was initially employed in clinical medicine for analysis of serum proteins. The electroimmuno diffusion was based on the principle that in a constant electric field and a given antibody dilution, the length of the precipitation cone formed is proportional to the concentration of the antigen. The method had a detection limit of 1.5 ng of enterotoxin A, and is stated to be rapid, simple, besides being quantitative.

2.6.3. immunofluorescent assay

The immunofluorescent method for detection of enterotoxin B in food smears and food extracts is another rapid method and could be completed within 4-5 h (Gasper *et al.*, 1973). The antibody is conjugated to fluorescein isothiocyanate and the method is able to detect the toxin as low as1 to0.05 μg/ml.

2.6.4. hemagglutination assay

Morse and Mah (1967) described a very sensitive microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B. Hemagglutination inhibition occurs when the enterotoxin antigen and antiserum were allowed to react and when sensitized red blood cells are added subsequently, there is no hemagglutination as long as the antibody has reacted with the antigen. The assay had advantages such as rapid results, small quantity of reactants, ease of reading, and reproducibility.

2.6.5. Enzyme immunoassay (EIA) and Enzyme linked immunosorbent assay (ELISA)

The need to develop a simple assay, which is non hazardous, requiring very less sample preparation, is cost effective and had other advantages like rapidity, sensitivity, and specificity lead to the development of methodologies involving enzyme labeling of the reaction components. Enzyme immune assays had the sensitivity which was equal to radioimmunoassays and did not have problems like lab safety, waste disposal, equipment expense, and short reagent life, which were encountered when RIA was employed. In 1969 the protocol for conjugation of enzymes to proteins was established

(Avrameas, 1969). As the procedure became routinely used, many areas of research and medicine began to employ the ELISA and EIA procedures for rapid and sensitive detection of both antigens and antibodies. described a double antibody solid-phase enzyme immune assay for detection of staphylococcal enterotoxins from foods. The sample preparation requires only 15 minutes and the entire assay is planned to be completed within 1-3 hours (Avrameas, 1969).

2.7. Detection of Enterotoxin genes

2.7.1. PCR

The detection of the enterotoxin genes of *S. aureus* was done mostly by PCR based methods. The nucleotide sequences of most of the enterotoxin genes are available and different degree of homology found among the genes lead to the designing of enterotoxin gene specific primers which allowed screening of samples for the presence of these genes. Firstly, Johnson *et al*., (1991) designed eight pairs of synthetic primers for detection of enterotoxins A-E, exfoliative toxins A and B, and TSST-1. Several set of primers were then proposed for fast multiplex PCR protocols. The primers targeted the internal regions of the toxin genes and the products were analyzed by gel electrophoresis. There are many reports on genotype analysis of enterotoxins by PCR (Gilligan *et al*., 2000; Mclauchlin *et al*., 2000; Omoe *et al*., 2002; Rosec and Giguad, 2002). Some variations and novelties have also been reported using the PCR based techniques.

Real–time PCR techniques are also gaining popularity among the methods that are currently available for the detection of enterotoxin genes. The advantage of real-time PCR is as the name suggests the amplification of the target could be monitored in real time. It is a quantitative method and it also eliminates the time consuming and laborious post PCR processes of amplicon detection by electrophoresis. Real-time fluorescence PCR for detection of enterotoxins A, B, C_1, and D was described by Klotz *et al*., (2003).

2.7.2. Microarrays

Microarrays can be used as a rapid means for the detection of enterotoxin genes. Though microarray based technologies are very expensive, and many laboratories do not opt for it due to cost constraints, the ability of the method to specifically and rapidly detect the presence of the enterotoxins genes is desirable. (Rosec and Giguad, 2002) reported a microarray-based one-tube assay for simultaneous detection of multiple enterotoxin genes of *S. aureus.*

Chapter Three

Materials and Methods

3.1. Study design

Descriptive cross sectional study

3.2. Study area

Study was conducted in Khartoum State the capital of Sudan. from the 1st of April till the 30th of May, 2017, The samples collected were from different hospitals (Omdurman military hospital, Police hospital, Soba hospital and Bahary hospital) representing Khartoum State.

3.3. Study population

Participants of this study were of all ages whom visited hospitals for care or for treatment of several illnesses. Both sexes were eligible for enrolment into the study and no age limits were set.

3.4.1. Inclusion criteria

From total of 80 samples, only 65 samples which positive for 16s housekeeping gene were enrolled to the study suffering from wound, urine or blood infection.

3.4.2. Exclusion criteria

Form 80 samples, 15 samples were negative for 16s housekeeping gene were excluded.

3.5. 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 prior the collection process.

3.6. Data collection

Data were collected using structured questionnaire (Appendix).

3.7. Laboratory work

3.7.1. Specimen collection and Cultivation

Mid stream urine was collected in a sterile containers for culture. An adequate amount of urine was taken on sterile dry Pasteur pipette for wet preparation. The labeled specimens were immediately placed on CLED agar. Specimens were incubated aerobically for 24 hours at 37°C.

Blood culture samples were collected after disinfecting the area by 70% alcohol, 5-10 ml of blood was collected and inculcate in blood culture bottle incubated at 37°C for at least 7 days and subculture on chocolate blood agar, blood agar and MacConkey agar.

Wound swap samples were collected after disinfecting the area by 70% alcohol, specimens were collected by sterile swap, were cultured on chocolate blood agar, blood agar and MacConkey agar at 37°C overnight.

3.7.2. Identification

Colonies were examined the next day. The organisms were identified according to the macroscopically morphology of the colonies

Gram Stain

The procedure was carried out according to Cheesbrough, (2006) as follows; 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 Bunzen 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 2 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 by oil lens 100X.

Biochemical tests DNAse, catalase, set of sugars and coagulase production tests were used to identify *Staphylococcus aureus* organisms (Collee *et al.*, 1996).

3.7.3. In- Vitro antibiotic sensitivity testing

Kirby-Bauer method was used (CLSI, 2007), the antibiotic discs used were from Himedia (Himedia Laboratories Pvt. Ltd, Mumbai 400086, India). The following antibiotics were used: Gentamicin (10mg), Ciprofloxacin (5mg), Vancomycin (30mg) and Methicillin (10mg).

The discs of the antibiotics were placed in the diagnostic susceptibility test agar (Muller Hinton Agar). 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 to (CLSI, 2007).

3.7.4. McFarland 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 of 2.35g of dehydrated barium chloride ($BaCl_2.2H_2O$) 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 sulphuric 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).

3.8. DNA Extraction

DNA was extracted by using Guanidine hydrochloride method (Oegema *et al*, 1979)

- 10ml of lysis buffer added to each sample and then samples were centrifuged for 5 min at 6000rpm.
- The above step was repeated 2 times until clear pallet appear.
- Supernatant was discard then 2 ml of white cell lysis buffer,1ml of Guanidine hydrochloride , 300 µl of NH4 acetate and 10 of proteinase K were added.
- 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 were centrifuged for 5 min at 6000rpm.
- Upper layer was collected to new falcon tube 10 ml of cold absolute ethanol was added to the samples then kept at -20 overnight.
- After overnight incubation samples were centrifuged for 10 min at 6000rpm then the supernatant was drained.
- Pellet was washed with 4 ml of 70% ethanol then was centrifuged for 10 min at 6000rpm.
- Supernatant was poured off and pellet was allowed to dry.
- Pallet was dissolve in 100 µl of sterile water for injunction and storage at -20.

DNA Purity

The purity of the extracted DNA was determined by spectrophotometer. And detection of DNA was done by running the DNA sample on 2% gel agarose (Sambrook *et al.*, 1989).

Preparation of 10 X TBE buffer

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water, pH 8.0 (Sambrook *et al*., 1989).

Preparation of 1X TBE buffer

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved (Sambrook *et al*., 1989).

Preparation of ethidium bromide

Ten milligrams of ethidium bromide powder were dissolved into 1 ml deionized water, and kept into brown bottle (Sambrook *et al*., 1989).

Preparation of agarose gel

Amount of 1 g of agarose powder dissolved by boiling in 50 ml 1X TBE buffer (2%), then was cooled to 55°C in water bath, then, 2 µl of (10mg\ml) Ethidium bromides were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed (Sambrook *et al*., 1989).

Molecular confirmation

All samples were confirmed as *S. aureus* by specific housekeeping gene primer (16s), negative samples were excluded.

3.9. Detection of *Staphylococcus aureus* Enterotoxin by Multiplex PCR

Multiplex PCR, amplification was done using TECHNE thermal cycler, DNA amplifies was done using Maxime PCR Premix kit (iNtRON, Korea),The PCR assay was carried out in a total volume of 20 µL of mixture containing 2 µL Maxime PCR Premix, 0.5 µL of each of the toxin gene-specific primers (5 µL), 5 µL of template DNA and 8 µL of

WFI (water for injection). The amplification conditions included three steps: heating at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec, and extension at 72°C for 30sec; and the final extension at 72°C for 7 min (Naresh *et al*., 2000).

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

Primer	Sequence 5' – 3'	Amplicon	Reference
SA-U[a]-F[b]	TGTATGTATGGAGGTGTAAC	-	Naresh *et al*., 2000
SA-A-R[c]	ATTAACCGAAGGTTCTGT	270	
SA-B-R	ATAGTGACGAGTTAGGTA	165	
ENT-C	AAGTACATTTTGTAAGTTCC	102	
SA-D-R	TTCGGGAAAATCACCCTTAA	306	
SA-E-R	GCCAAAGCTGTCTGAG	213	

a: Universal, b: Forward, c: Reverse.

3.10. Visualization of the DNA product

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface,10 µl of PCR products from each samples was added to wells of electrophoreses, 5 µl of 50-bp DNA ladder (iNtRON, Korea),was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V transilluminater and photographed (Uvitec – UK), (Naresh *et al*., 2000).

3.11. Statistical Analysis

All results achieved in the study were analyzed. Data were introduced to Scientific Package of Social Sciences (SPSS) software to estimate the *p* value of significance using Chi square test, means and averages were also calculated.

Chapter Four

RESULTS

Total number of 65 samples positive for 16s housekeeping gene were enrolled in this study. 56 were positive for one or more *Staphylococcus aureus* toxin genes. While 9 isolates were negative for all toxin genes. Infected wound samples 30/65 (47%), Urine samples 25/65 (38%), blood samples 10/65 (15%).

4.1. The association between the *S.aureus* Enterotoxin genes and Age group

Most of study population were females 36/65 (56%), while males were 29/65 (44%), their age ranged from 10 to 70 with mean 40 years, and most of them were in the 31 – 40 years as indicated in Table (2)

Table (2) Age group of enrolled patients and relationship with *S.aureus* Enterotoxin genes

Genes	**10-20**	**21-30**	**31-40**	**41-50**	**51-60**	**61-70**	**P-value**
SA-A	2	0	1	1	1	0	0.463
SA-B	3	3	9	3	5	2	0.096
ENT-C	4	7	4	2	4	1	0.752
SA-E	1	1	1	1	0	0	0.929
Total	10	11	15	7	10	3	

4.2. The association between the *S.aureus* Enterotoxin genes and gender

The overall results revealed that total of 36 females, 3/36 (8%) positive for *SA-A* gene, 14/36 (39%) positive for *SA-B* gene, 14/36 (39%) positive for *ENT-C* gene, 3/36 (8%) positive for *SA-E* gene, *SA-D* gene was negative for all samples, while males are 29, 2/29 (7%) positive for *SA-A* gene, 11/29 (40%) positive for *SA-B* gene, 8/29 (28%) positive for *ENT-C* gene, 1/29 (3%) positive for *SA-E* gene, *SA-D* gene was negative for all samples. The results show no significant association between the *S.aureus* Enterotoxin genes and gender as indicated in Table (3)

Table (3) The association between the *S.aureus* Enterotoxin genes and gender

Genes	*SA-A*		*SA-B*		*ENT-C*		*SA-E*		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Female	3	33	14	22	14	22	3	33	36
Male	2	27	11	18	8	21	1	28	29
Total	5	60	25	30	22	43	4	61	65
P-value	0.829		0.937		0.338		0.415		

4.3. The association between the *S.aureus* Enterotoxin genes and clinical samples

The overall results revealed that total of 65 clinical samples, 30/65 (46%) were from infected wound, followed by 25/65 (38%) urine samples and 10/65 (15%) blood samples. There was no significant association between the presence of the *S.aureus* Enterotoxin genes (*SA-B*, *ENT-C, SA-E*) P-value (0.949, 0.332, 0.444) and clinical samples, but there is significant strong association between the presence *S.aureus* Enterotoxin gene (*SA-A*) P-value (0.000) and blood samples as indicated in Table (4)

Table (4) The association between the *S.aureus* Enterotoxin genes and clinical samples

Genes	***SA-A***		***SA-B***		***ENT-C***		***SA-E***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Wound swap	0	30	12	18	9	21	3	27	30
Urine	0	25	9	16	11	14	1	24	25
Blood	5	5	4	6	2	8	0	10	10
Total	5	60	25	40	22	43	4	61	65
P-value	0.000		0.949		0.332		0.444		

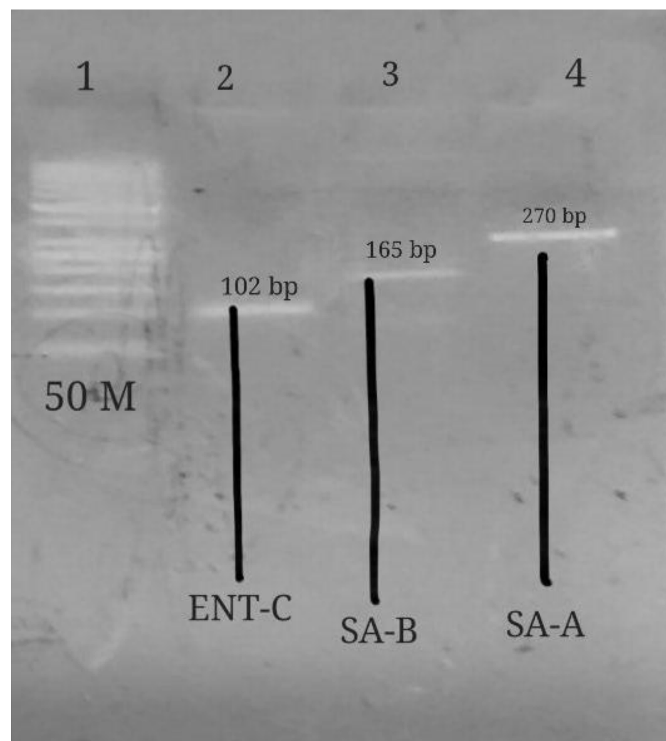

Fig (1) Agarose gel electrophoresis of multiplex PCR product, 1= 50 pb ladder, 2= positive *ENT-C* gene, 3= positive *SA-B* gene, 4= positive *SA-A* gene

4.4. The association between the *S.aureus* Enterotoxin genes and Antibiotic Mithecillin

The overall results revealed that total of 36/65 (55%) samples sensitive for Mithecillin. There was no significant association between the presence of the *S.aureus* Enterotoxin genes (*SA-A, SA-B, ENT-C, SA-E*) P-value (0.471, 0.554, 0.667, 0.415) to Mithecillin antibiotic, 2/36 (6%) sensitive for *SA-A* gene, 15/36 (42%) sensitive for *SA-B* gene, 13/36 (36%) sensitive for *ENT-C* gene, 3/36 (8%) sensitive for *SA-E* gene as indicated in Table (5)

Table (5) The association between the *S.aureus* Enterotoxin genes and Mithecillin

Genes	***SA-A***		***SA-B***		***ENT-C***		***SA-E***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	2	34	15	21	13	23	3	33	36
Resistance	3	26	10	19	9	20	1	28	29
Total	5	60	25	40	22	43	4	61	65
P-value	0.471		0.554		0.667		0.415		

Vancomycin

The overall results revealed that total of 25/65 (38%) samples sensitive for Vancomycin. There was no significant association between the presence of the *S.aureus* Enterotoxin genes (*SA-A, SA-B, ENT-C, SA-E*) P-value (0.377, 0.058, 0.407, 0.568) to Vancomycin antibiotic, 1/25 (4%) sensitive for *SA-A* gene, 6/25 (24%) sensitive for *SA-B* gene, 10/25 (40%) sensitive for *ENT-C* gene, 1/25 (4%) sensitive for *SA-E* gene as indicated in Table (6)

Table (6) The association between the *S.aureus* Enterotoxin genes and Vancomycin

Genes	**SA-A**		**SA-B**		**ENT-C**		**SA-E**		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	1	24	6	19	10	15	1	24	25
Resistance	4	36	19	21	12	28	3	37	40
Total	5	60	25	40	22	43	4	61	65
P-value	0.377		0.058		0.407		0.568		

Gentamicin

The overall results revealed that total of 27/65 (42%) samples sensitive for Gentamicin. There was no significant association between the presence of the *S.aureus* Enterotoxin genes (*SA-A, SA-B, ENT-C, SA-E*) P-value (0.942, 0.061, 0.647, 0.488) to Gentamicin antibiotic, 2/27 (7%) sensitive for *SA-A* gene, 14/27 (52%) sensitive for *SA-B* gene, 10/27 (37%) sensitive for *ENT-C* gene, 1/27 (7%) sensitive for *SA-E* gene as indicated in Table (7)

Table (7) The association between the *S.aureus* Enterotoxin genes and Gentamicin

Genes	***SA-A***		***SA-B***		***ENT-C***		***SA-E***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	2	25	14	13	10	17	1	26	27
Resistance	3	35	11	27	12	26	3	35	38
Total	5	60	25	40	22	43	4	61	65
P-value	0.942		0.061		0.647		0.488		

Ciprofloxacin

The overall results revealed that total of 29/65 (45%) samples sensitive for ciprofloxacin. There was no significant association between the presence of the *S.aureus* Enterotoxin genes (*SA-A, SA-B, ENT-C, SA-E*) P-value (0.829, 0.937, 0.338, 0.415) to ciprofloxacin antibiotic, 2/29 (7%) sensitive for *SA-A* gene, 11/29 (38%) sensitive for *SA-B* gene, 8/29 (28%) sensitive for *ENT-C* gene, 1/29 (4%) sensitive for *SA-E* gene as indicated in Table (8)

Table (8) The association between the *S.aureus* Enterotoxin genes and Ciprofloxacin

Genes	***SA-A***		***SA-B***		***ENT-C***		***SA-E***		**Total**
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Sensitive	2	27	11	18	8	21	1	28	29
Resistance	3	33	14	22	14	22	3	33	36
Total	5	60	25	40	22	43	4	61	65
P-value	0.829		0.937		0.338		0.415		

Chapter Five

5.1. Discussion

The results of the present study showed higher frequency of *SA-B* gene compared to the rest of the genes (38%), which may indicated an essential role of the virulence gene in *Staphylococcus aurous* causing different infections among Sudanese patients. These results was not agree with most published reports, this findings was totally agreed with (in Sudan) Mohammed *et al*., (2014) who found that (35%) *SA-B* gene positive, but not agree with Manfredi *et al*., (2010) who found that (15%) *SA-B* gene positive and Rall *et al*.,(2008) who found that (8%) *SA-B* gene positive. Unlike most toxins, SEs are not directly cytotoxic and cell entry is not a requirement for them to cause an effect. The Centers for Disease Control and Prevention (CDC) place one SE, staphylococcal enterotoxin B (*SA-B*), as a select agent based on its universal availability, ease of production and dissemination, and the potential to cause moderate but widespread illnesses. Additionally, because these agents are common to the environment and the diseases they cause are similar to other diseases, Category B agents require close environmental monitoring and enhanced disease surveillance (Hale, 2012).

The result show (8%) *SA-A* gene positive, this findings was totally agreed with Rall *et al*.,(2008) who found that (5%) *SA-A* gene positive, but not agreed with (in Sudan) Mohammed *et al*., (2014) who found that (38%) *SA-A* gene positive, Manfredi *et al*., (2010) who found that (55%) *SA-A* gene positive and Tang *et al*., (2012) who found that (36%) *SA-A* gene positive. The result show significant association (P-value 0.000) between blood samples and *SA-A* gene, The Toxic Shock Syndrome Toxin (TSST) and the enterotoxins produced by *S. aureus* have superantigenic activity. Superantigens stimulate T cells non-specifically without any specific antigen recognition. In a normal antigenic response only one T cell in a population of about 10,000 is stimulated, where as if a superantigen is involved

one in every five T cells is stimulated leading to an enormous release of cytokines. The superantigens bind T cell receptors and major histocompatibility complex (MHC) class II molecules outside the peptide binding groove (Bohach *et al.,* 1990). Even minute quantities of superantigens can lead to massive stimulation of T cells. About 100 pg/ml of superantigen is enough to trigger a response (Bohach *et al.,* 1990). As a result of non-specific proliferation, large populations of effector CD4 cells begin to stimulate the monocytes resulting in the production of cytokines like IL-1 and TNF α . Since large numbers of cells are involved, instead of localized secretion of these cytokines, a systemic secretion occurs. During an infection only localized secretion occurs, where as the superantigen mediated stimulation results in a massive systemic response (Schlievert, 1993).

The result show (33%) *ENT-C* gene positive, this result is not far from Khudor *et al*, (2012) who found (25%) *ENT-C* gene positive, Rall *et al*.,(2008) who found that (25%) *ENT-C* gene positive, but not agree with Manfredi *et al*., (2010) who found that (7%) *ENT-C* gene positive.

The result show (6%) *SA-E* gene positive, this findings was totally agreed with Rall *et al*.,(2008) who found that (5%) *SA-E* gene positive. *SA-D* gene was not detected and this result agree with Khudor *et al*, (2012).

There was 9 samples were negative for all *Staphylococcus aureus* enterotoxin genes these negative isolates may be are normal flora. In addition to many virulence determinants contribute to the pathogenicity of *S. aureus*, they are also the products of different genes which can be detected by PCR. However there is always the possibility of mutation at the level of the corresponding gene, leading to the absence of its detection. Therefore a positive PCR shows the presence of the enterotoxin genes but a negative PCR does not point the absence of the corresponding operon (Tarchouna *et al*., 2013).

The present study result showed that the *S. aureus* strains producing enterotoxins isolated in Sudan have a different toxins profile compared

with other studies and it seems that the *S. aureus* strains depends on the regional geography and climate. It is believed that the epidemiology and prevalence of *S. aureus* strains producing enterotoxins among Sudanese patients are different from other countries. Perhaps some factors such as customs, food diets, public health, and even methods of sampling have great rules in prevalence of *S. aureus* producing enterotoxins strains (Naresh *et al*., 2000).

5.2. Conclusion

The study concluded that:

- *SA-B* gene is found predominant
- *SA-D* gene is not detected
- No significant association between S.aureus enterotoxin genes and (age group, gender and antimicrobial susceptibility testing)
- Found significant association between *SA-A* gene and blood samples
- Multiplex PCR was satisfactory for detection UPCE virulence genes

5.3. Recommendations

1. Large sample size is recommended to detection of *S.aureus* enterotoxin genes.
2. Further studies are needed to detection of *S.aureus* enterotoxin genes and to determine the physiopathology of these infection to find possible prevention measures.
3. Using more advance molecular techniques for detection of *S.aureus* enterotoxin genes.

REFERENCES

Argudin, M. A.; Mendoza, M. C. and Rodicio, M. R. (2010). Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins* **2**: 1751-1773.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of conjugates for the detection of antigens and antibodies. *Immunochemistry*. **6**:43-52.

Barber, M. (1961) Methicillin-resistant staphylococci. *Journal of Clinical Pathology* 14, 385–93

Balaban, N., and Rasooly, A. (2000). Staphylococcal enterotoxins. Int. J. Food *Microbiol*. 61, 1–10.

Bohach, G.A., D. J. Fast, R. D. Nelson, and P. M. Schlievert. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Crit. *Rev. Microbiol*. 17(4):251-272.

Cafferkey, M.T., Hone, R., Coleman, D. et al. (1985) Methicillin-resistant *Staphylococcus aureu*s in Dublin 1971–84. Lancet 2, 705–8.

Casman, E. P., and R. W. Bennett. (1964). Production of antiserum for staphylococcal enterotoxin. *Appl. Microbiol*. **12**:363-367.

Centers for Disease Control and Prevention (2002a) *Staphylococcus aureus* resistant to vancomycin—United States, 2002. MMWR. 51, 565–7.

Centers for Disease Control and Prevention (2000b) Vancomycin-resistant *Staphylococcus aureus*—Pennsylvania, 2002. MMWR. 51, 902.

Cheesbrough M. (2006). District Laboratory Practice in Tropical Countries, Part2, Cambridge University Press, United Kingdom, PP. 60-64.

Chemie AG. (2014).Microbiology Culture Media Manual, Fifth edition, Pages (40, 83, 114, 166, 191, 195).

Chen, T.R.; Chiou, C.S. and Tsen, H.Y. (2004). Use of novel PCR primers specific to the genes of staphylococcal enterotoxin G, H, I for the survey of *Staphylococcus aureus* strains isolated from food-poisoning cases and food samples in Taiwan. *Int. J. Food Microbiol*. **92**: 189–197.

Clinical and Laboratory Standards Institute (CLSI), (2007) Interpretive chart of zonesizes Performance Standards for Antimicrobial Susceptibility Testing, SeventeenthInformational Supplement.USA, 27 (1), 32-52.

Collee, J. G., Duguid, J. P., Fraser, A. G. and Marmion, B. P. (1996) Laboratorystrategies in the diagnosis of infectious syndromes. InPractical MedicalMicrobiology, eds Collee, J. G., Barrie, M. P., Fraser, A.G. and Simons, A., 14thed. Churchill Livingstone, New York, 53-93.

Cookson, B.D., Phillips, I. (1988) Epidemic methicillin-resistant *Staphylococcus aureus.* The Journal of Antimicrobial Chemotherapy 21(Suppl. C), 57–65.

Cowan, S.T., and Steel, K.L.(1964). Comparison of differentiating criteria for staphylococci and micrococci. J. Bacteriol. 88, 804–805.

Cunha, M. L. R. S. (2009). Pathogenesis of *Staphylococcus aureus* and Bovine Mastitis. *Int. J. Med. Biol. Frontiers.* **15**: 1031-1042.

Daum, R.S., Ito, T., Hiramatsu, K. et al. (2002) A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. *The Journal of Infectious Diseases* 186, 1344–7.

Derzelle, S.; Dilasser, F.; Duquenne, M. and Deperrois, V. (2009). Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. *Food Microbiol*. **26**: 896–904.

Devriese, L. A., Poutrel, B., Kilpper-Balz, R. and Schleifer, K. F. (1983). *Staphylococcus gallinarum* and *Staphylococcus caprae*, two new species from animals. *Int. J. Syst. Bacteriol*. **33**: 480-486.

Dinges, M.M., Orwin, P.M., Schlievert, P.M. (2000) Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews* 13, 16–34.

Duckworth, G.J., Lothian, J.L., Williams, J.D. (1988) Methicillin-resistant *Staphylococcus aureus*: report of an outbreak in a London teaching hospital. *The Journal of Hospital Infection* 11, 1–15.

Ferens, W.A., Davis, W.C., Hamilton, M.J., Park, Y.H.,Deobald, C.F., Fox, L., and Bohach, G. (1998). Activation of bovine lymphocyte subpopulations by staphylococcal enterotoxin C. Infect. Immun. 66, 573–580

Fowler, V.G. Jr, Olsen, M.K., Corey, G.R. et al. (2003) Clinical identifiers of complicated *Staphylococcus aureus* bacteremia. *Archives of Internal Medicine* 163, 2066–72.

Gasper, E., C. Heimsch, and A.W. Anderson. (1973). Quantitative detection of type A staphylococcal enterotoxin by Laurell electroimmunodiffusion. *Appl. Microbiol*. **25**:421-426.

Gillespie, M.T., Lyon, B.R., Messerotti, L.J., Skurray, R.A. (1987) Chromosomeand plasmid-mediated gentamicin resistance in *Staphylococcus aureus* encoded by Tn4001. *Journal of Medical Microbiology* 24, 139–44.

Gilligan, K., M. Shipley, B. Stiles, T. L. Hadfield, and M. Sofi Ibrahim. (2000). Identification of *Staphylococcus aureus* enterotoxins A and B genes by PCR-ELISA. *Mol. Cell. Probes* **14**:71-78.

Gundogan, N.; Citak, S. and Turan, E. (2006). Slime production, DNAse activity and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk, pasteurised milk and ice cream samples. *Food Control* .**17**:389-392.

Hale, M. L. (2012). Staphylococcal Enterotoxins, Staphylococcal Enterotoxin B and Bioterrorism. In Bioterrorism. InTech.

Hall, H. E., R. Angelotti, and K. H. Lewis. (1965). Detection of the staphylococcal enterotoxins in food. *Health Lab. Sci*. **2**:179-191.

Havelaar, A.H.; Brul, S.;de Jong, A.; de Jonge, R.; Zwietering, M.H. and Ter Kuile, B.H. (2010). Future challenges to microbial food safety. *Int J Food Microbiol*. **139**:79-94.

Hennekinne, J.A.; Ostyn, A.; Guillier, F.; Herbin, S.; Prufer, A. L. and Dragacci, S. (2010). How Should Staphylococcal Food Poisoning Outbreaks Be Characterized. *Toxins*. **2**: 2106-2116.

Hiramatsu, K., Aritaka, N., Hanaki, H. et al. (1997a) Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350, 1670–3.

Hiramatsu, K., Hanaki, H., Ino, T. et al. (1997b) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *The Journal of Antimicrobial Chemotherapy* 40, 135–6.

Hiramatsu, K. (2001) Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. Lancet Infectious Diseases 1, 147–55.

Ikeda, T.; Tamate, N.; Yamaguchi, K. and Makino, S. (2005). Mass Outbreak of Food Poisoning Disease Caused by Small Amounts of Staphylococcal Enterotoxins A and H. *Appl. Environ. Microbiol.* **71**: 2793–2795

Jevons, M.P. (1961) 'Celbenin'-resistant staphylococci. British Medical Journal 1, 124–5.

Johnson, W. M., S. D. Tyler, E. P. Ewan, F. E. Ashton, D. R. Pollard, and K. R. Rozee. (1991). Detection of genes for enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J. Clin. Microbiol*. **29**:426-430.

Karahan, M. and Cetinkaya, B. (2007). Coagulase gene polymorphism detected by PCR in *Staphylococcus aureus* isolated from subclinical bovine mastitis in Turkey. *The Veterinary Journal*. **174**: 428-431..

Kayser, F.H; Bienz,K.A.; Eckert, J. and Zinkernagel, R.M.(2005). Color Atlas of Medical Microbiology.11th ed. Thieme Verlag, NewYork, USA: 300-303.

Khudor, M. H., Abbas, B. A., & Idbeis, H. I. (2012). Detection of enterotoxin genes of *Staphylococcus aureus* isolates from raw milk. Bas. J. Vet. Res, 11(1), 254-264.

Kilpper-Balz, R. and Schleifer, K. H. (1981). Transfer of *Peptococcus saccharolyticus* Foubert and Douglas to the genus *Staphylococcus*: *Staphylococcus saccharolyticus* (Foubert and Douglas) comb nov. Zentralbl. Bakteriol. Hyg. Abt. 1Orig. Reihe : 324-331.

Kloos, W.E. and Schleifer, K. H. (1975). Isolation and characterization of staphylococci from human skin. II. Descriptions of four new species *Staphylococcus warneri, Staphylococcus capitis, Staphylococcus hominis,* and *Staphylococcus simulans*. *Int. J. Syst. Bacteriol*. **25**: 62-79.

Kloos,W. E. and Wolfshohl, J. F. (1991). *Staphylococcus cohnii subspecies Staphylococcus cohnii subsp. cohnii subsp. nov.* and *Staphylococcus cohnii subsp urealyticum subsp. nov*. *Int. J. Syst. Bacteriol*. **41**: 284- 289.

Klotz, M., S. Opper, K. Heeg, and S. Zimmermann. (2003). Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. *J. Clin. Microbiol*. **41**(10): 4683-4687.

Larkin, E.A.; Carman, R.J.; Krakauer, T. and Stiles, B.G. (2009). *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. *Curr. Med. Chem*. **16**: 4003-4019.

Larsen, H.D., Aarestrup, F.M., and Jensen, N.E. (2002). Geographical variation in the presence of genes encoding superantigenic exotoxins and β-hemolysin among *Staphylococcus aureus* isolated from bovine mastitis in Europe and USA. Vet. Microbiol. 85, 61–67.

Laurell, C. B. (1966). Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem*. **15**:45-52.

Le Loir, I.; Baron, F. and Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res*. **2**: 63-76.

Ler, S.G.; Lee, F.K. and Gopalakrishnakone, P. (2006). Trends in Detection of Warfare Agents. Detection Methods for Ricin, Staphylococcal Enterotoxin B and T2 Toxin.*J. Chromatogr. A.* 1133:1-12.

Leung, D. Y., Meissner, H. C., Fulton, D. R., Murray, D. L., Kotzin, B. L. and Schlievert, P. M. (1993). Toxic shock syndrome toxin-secreting *Staphylococcus aureus* in Kawasaki syndrome. *Lancet*. **342**(8884): 1385-1388.

Lina, G.; Bohach, G.A.; Nair, S.P.; Hiramatsu, K.; Jouvin-Marche, E. and Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J. Infect. Dis*. **189**: 2334–2336.

Lindsay, J.A.; Ruzin, A.; Ross, H.F.; Kurepina, N. and Novick, R.P. (1998). The Gene for Toxic Shock Toxin Is Carried by a Family of Mobile Pathogenicity Islands in *Staphylococcus aureus. Mol. Microbiol.* **29**: 527–543.

Lisa R.W. (2004). *Staphylococcus aureus* exfoliative toxins: How they cause disease. *Derm. Foundat*. **122**: 1070-1077.

Liu, C., Chambers, H.F. (2003) *Staphylococcus aureus* with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. *Antimicrobial Agents and Chemotherapy* 47, 3040–5.

Lyon, B.R., Skurray, R. (1987) Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. *Microbiological Reviews* 51, 88–134.

Manfredi, E. A.; Leotta, G. A. and Rivas, M. (2010). Multiplex PCR for the detection of sea, seb, sec, sed and see genes of *Staphylococcus aureus*. Characterization of isolates from food. *Rev Argent Microbiol*. **42**(3): 212-215.

Mclauchlin, J., G.L. Narayanan, V. Mithani, and G. O'Neill. (2000). The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. *J. Food Prot*. **63**: 479-488.

Mohammed, T. A., Khalid, A. E., and Mohamed, A. (2014) PCR detection of staphylococcal enterotoxin A and B Genes in *staphylococcus aureus* isolated from salted fermented fish. *Microbiology journal*, 4: 51, 56.

Morandi, S.; Brasca, M.; Andrighetto, C.; Lombardi, A. and Lodi, R. (2009). Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Strains from Italian Dairy Products. *Int. J. Microbiol*. 501362:1–501362:7.

Morse, S.A., and R.A. Mah. (1967). Microtiter hemagglutination-inhibition assay for staphylococcal enterotoxin B. *Appl. Microbiol*. **15**(1): 58-61.

Muller-Alouf, H., C. Carnoy, M. Simonet and J. E. Alouf. (2001). Superantigen bacterial toxins: state of the art. *Toxicon*. **39**:1691-1701.

Naresh, K.; Sharma, D.; Catherine, E.; Rees, D. and Christine, E. R. (2000). Development of a single-reaction multiplex PCR toxin typing assay for *staphylococcus aureus* strains. *Appl and Environ. Microbiol*. **66**(4):1347-1353.

Oegema, T. R., Hascall, V. C., & Eisenstein, R. (1979). Characterization of bovine aorta proteoglycan extracted with guanidine hydrochloride in the presence of protease inhibitors. Journal of Biological Chemistry, 254(4), 1312-1318.

Ogston, A. (1880). Uber Abscesse. Archiv fur Klinische Chirurgie 25, 588–600.

Ogston, A. (1882). Micrococcus poisoning. J. Anat. Physiol. 171054, 24–58.

Okuma, K., Iwakawa, K., Turnidge, J.D. et al. (2002) Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. *Journal of Clinical Microbiology* 40, 4289–94.

Omoe, K., M. Ishikawa, Y. Shimoda, D.L. Hu, S. Ueda and K. Shinagawa. (2002). Detection of seg, seh and sei genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S.aureus* isolates harboring seg, seh, or sei genes. *J. Clin. Microbiol*. **40**: 857-862.

Panton, P.N., and Valentine, F.C.O. (1932). STAPHYLOCOCCAL TOXIN. The Lancet 219, 506–508.

Partida, A.; Espunes,T.; Marinez, J.B. (2010). Characterization and Persistence of *Staphylococcus aureus* Strains isolated from the Anterior Nares and Throats of Healthy Carriers in a Mexican Community. *J. of Clin. Microbiol*. **48**(5): 1701-1705.

Pasteur, L. (1880). De l'extension de la theorie des germes a l'etiologie de queques maladies communes. C.R. Seances Soc. Biol. Paris. 90, 1035–1044.

Pavillard, R., Harvey, K., Douglas, D. et al. (1982) Epidemic of hospital-acquired infection due to methicillin-resistant *Staphylococcus aureus* in major Victorian hospitals. *Medical Journal of Australia* 1, 451–4.

Pesavento, G.; Ducci, B.; Comodo, N. and Nostro, A.L. (2007). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw meat: a research for methicillin resistant *Staphylococcus aureus* (MRSA). *Food Control.* **18**:196-200.

Pinchuk, I. V.; Beswick, E. J. and Reyes, V. E. (2010). Staphylococcal Enterotoxins. *Toxins*. **2**: 2177-2197.

Rall, V. L. M., Vieira, F. P., Rall, R., Vieitis, R. L., Fernandes, A., Candeias, J. M. G., ... & Araujo, J. P. (2008). PCR detection of staphylococcal enterotoxin genes in *Staphylococcus*

aureus strains isolated from raw and pasteurized milk. *Veterinary Microbiology*, 132(3), 408-413

Rosec, J. P., and O. Gigaud. (2002). Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *Int. J. Food Microbiol*. **77**:61-70.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989).Gel electrophoresis of DNA. In: *Molecular Cloning: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schaefler, S., Jones, D., Perry, W. et al. (1981) Emergence of gentamicin- and methicillin-resistant *Staphylococcus aureus* strains in New York City hospitals. *Journal of Clinical Microbiology* 13, 754–9.

Schlievert, P. M. (1993). Role of superantigens in human disease. J. Infect. Dis.167:997-1002.

Schlievert, P. M. and Case, L. C. (2007). Molecular analysis of staphylococcal superantigens. *Methods Mol. Biol.* **391**: 113-126.

Shalita, Z., Murphy, E., Novick, R.P. (1980) Penicillinase plasmids of *Staphylococcus aureus*: *structural and evolutionary relationships. Plasmid* 3, 291–311.

Shanson, D.C., Kensit, J.C., Duke, R. (1976) Outbreak of hospital infection with a strain of *Staphylococcus aureus* resistant to gentamicin and methicillin. Lancet 2, 1347–8.

Sila, J.; Sauer, P.; Kolar, M. (2009). Comaprison of the prevalence of genes coding for enterotoxins, exfoliatins, Panton-Valentine leukocidin and TSST-1 between methicillin-resistant and methicillin-susceptible isolates of *Staphylococcus aureus* at the University Hospital in Olomouc. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub*.**153**: 215–218.

Spaan, A.N., Surewaard, B.G., Nijland, R., and van Strijp, J.A. (2013). Neutrophils versus *Staphylococcus aureus*: a biological tug of war. Annu Rev Microbiol. 67, 629–650.

Tang, J; Tang, C., Wang, Y., Chen, J.; Liu, J.; Liu, L. and Yue, H. (2012). Surveillance study of enterotoxin genes in *Staphylococcus aureus* isolates from goats of different slaughterhouses in Sichuan, China. *Annals of Microbiology*. 62: 1247-1253.

Tarchouna M, Ferjani A, Selma WB, Boukadida J. (2013) Distribution of uropathogenic virulence genes in *Escherichia coli* isolated from patients with urinary tract infection. Int J Infect Dis17:450-3.

Tollersrud, T., Kampen, A.H., and Kenny, K. (2006).*Staphylococcus aureus* enterotoxin D is secreted in milk and stimulates specific antibody responses in cows in the course of experimental intramammary infection. Infect. Immun. 74, 3507–3512.

Varshney, A. K.; Mediavilla, J. R.; Robiou, N.; Guh, A.; Wang, X.; Gialanella, P.Levi, M. H. Kreiswirth, B. N. and Fries, B.C. (2009). Diverse Enterotoxin Gene Profiles Among Clonal Complexes of *Staphylococcus aureus* isolates From the Bronx, New York. *Appl. Environ. Microbiol*. **75**: 6839–6849.

Von Eiff, E. C.; Becker, K.; Machka, K.; Stammer, H. and Peters, G. (2001). Nasal Carriage as a Source of *Staphylococcus aureus* Bacteremia. Study Group. *N. Engl.J. Med*. **344**: 11–16.

Vos, P.; Garrity, G.; Jones, D.; Krieg, N. R.; Ludwig, W.; Rainey, F.A.; Schleifer K. H. and Whitman, W. B. (2009). The Firmicutes In: Bergey's manual of determinative bacteriology. 2nd ed. Volume 3. Springer, New York

Yamaguchi, T.; Hayachi, T.; Takami, H.; Nakasome, K.; Ohnish, M.; Nakayama,K.; Yamada, S.; Komatsuzawa, H. and Sugai, M. (2000). Phage conversion ofesfoliative toxin A production in *Staphylococcus aureus*. *Mol. Microbiol*. **38**: 694-705.

Yoshizawa, Y.; Sakurada, J.; Sakurai, S.; Machida, K.; Kondo, I. and Masuda, S. (2000). An exfoliative toxin A-converting phage isolated from *Staphylococcus aureus* strain ZM. *Microbiol. Immunol*. **44**: 189-191

APPENDICES

Appendix I – questionnaire

Sudan University of Science and Technology

College of Graduate studies

Molecular Detection of *Staphyloccus aureus* toxin Genes Isolated from Different Clinical Samples in Khartoum State

By: Lemya Hassan Mahmoud

Supervised by: Prof. Yousif Fadlallah Hamedelnil

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…………………………………..

Signature:………………………………………………………..

Appendix II

Figure (1) Catalase test for identification of *Staphylococcus* species

Figure (2) manitol fermentation test for identification of *Staphylococcus* aureus

Figure (3) DNAes test for identification of *Staphylococcus* aureus

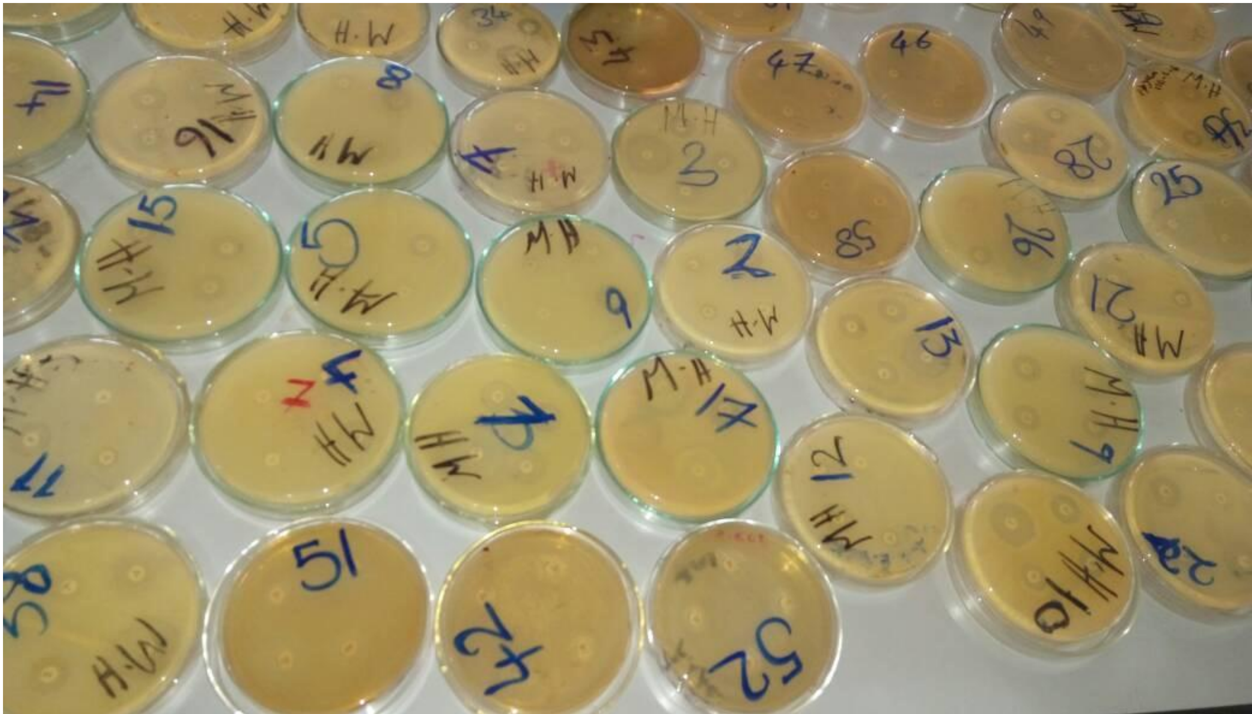

Figure (4) Antimicrobial susceptibility testing

Figure (5) Microcenterfuge device

Figure (6) Thermocycle device

Figure (7) gel electrophoresis and power supply device

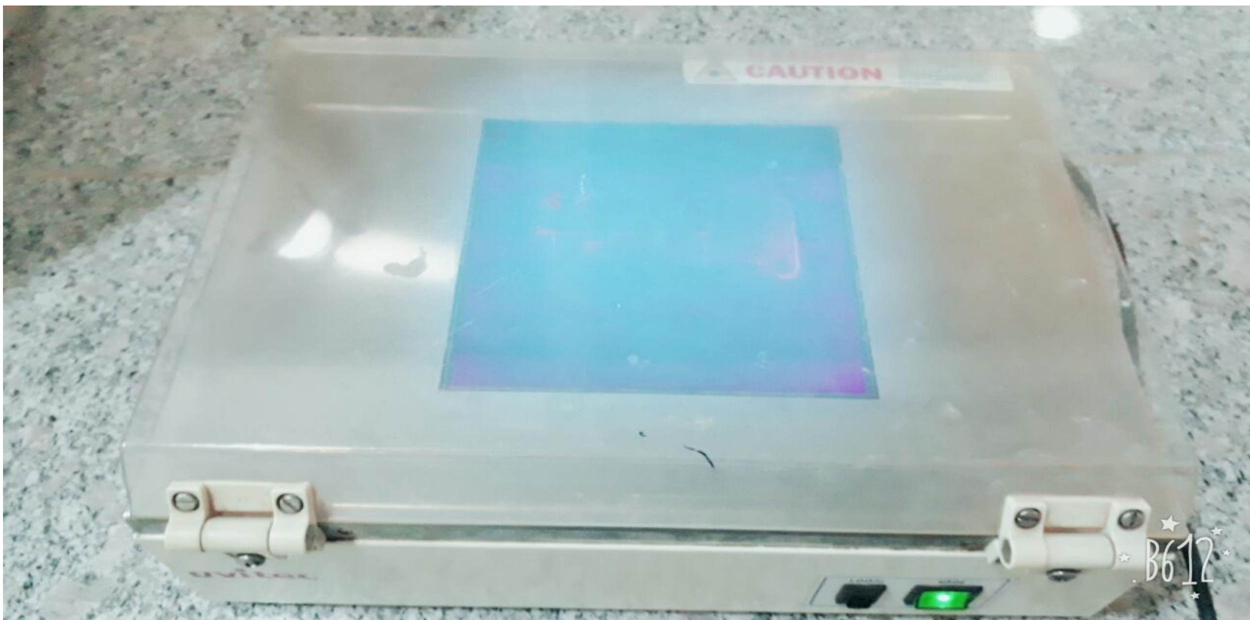

Figure (8) UV Light transilluminater device