REPUBLIC OF TURKEY
AYDIN ADNAN MENDERES UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF MOLECULAR BIOTECHNOLOGY
MOLECULAR BIOTECHNOLOGY MASTER'S PROGRAM

CLONING OF BETA HEMOLYSIN GENE FROM COAGULASE NEGATIVE STAPHYLOCOCCI AND TESTING OF BIOLOGICAL ACTIVITY

SAHD ALI
MASTER'S PROGRAM THESIS

SUPERVISOR
Prof. Dr. Gamze BAŞBÜLBÜL

This thesis was prepared and supported by Aydın Adnan Menderes University Scientific Research Projects Unit FEF-19021 carried out in Recombinant Protein and Research (REDPROM) laboratory.

AYDIN–2021

ACCEPTANCE AND APPROVAL FORM

The thesis with the title of "Cloning of Beta Hemolysin gene from Coagulase-negative staphylococci and Testing of Biological Activity" prepared by Sahd ALI, Master Student at the Graduate school of Health Sciences Program at the Department of Molecular Biotechnology was accepted by the jury members whose names and titles are presented below as a result of thesis defense on 07/01/2021.

Thesis defence date: 07/01/2021

(Supervisor) : Prof.Dr. Gamze BAŞBÜLBÜL Aydın Adnan Menderes University .....

Member : Prof.Dr. Bülent BOZDOĞAN Aydın Adnan Menderes University …….....

Member : Dr. Lecturer Tuğrul HOŞBUL University of Health Sciences .....

APPROVAL:

This Master Thesis is approved by the jury above in accordance with the relevant articles of the Aydın Adnan Menderes University Graduate Education and Examination Regulations and was accepted by the Board of Directors decision number…….…. taken at the session number ……. of the Institute of Health Sciences on the date ……..……..

Prof.Dr. Süleyman AYPAK

Institute Director

ACKNOWLEDGEMENT

My foremost and sincere appreciation goes to my supervisor, Prof. Dr. Gamze BAŞBÜLBÜL, for the help, patient and time that she spent in the course of making this thesis a successful one. It was because of her leadership skills, confident, encouragements, humanitarian and moral values that have brought me so far in my studies.

I would like to also acknowledge my program coordinator, Prof. Dr. Bülent BOZDOĞAN, for making this Molecular Biotechnology cross boarder curriculum a unique one. His work and dedication demonstrated to me which concern for global affairs supported by an ‘‘engagement’’ in comparative leterature and modern technology should always transcend academia and provide a quest of our time.

I would like to thank Aydin Adnan Menderes University Rectorate (ADÜ-BAP: FEF-19021), who provided a financial resources for the realization of this study.

In addition, a great thank you to Dr. Lecturer Tuğrul HOŞBUL for being a jury member in my thesis defence. A big thanks to Dr. Erman ORYASIN and to my esteemed REDPROM laboratory colleagues; whom did not withhold their help, love and time by encouraging me during my laboratory work. They have been ever ready in assisting me and this have made our discipline a successful one.

All this would not have been possible, if my family hadn’t been my backbone. I expressed a heart full of grattitude to you all. My lovely mom and siblings, I so much treasure your endless love, material and moral support you gave me during this period of study.

Sahd ALI

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AXI	Ampisilin, X-gal, IPTG
β	Beta
hlb	Beta hemolysin
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
HAI	Hospital acquired infections
IPTG	Isopropyl β-d-1-thiogalactopyranoside
Kd	Kilodalton
MSCRAMM	Microbial surface components adhesive matrix molecules
µl	Microlitre
MGE	Mobile genetic elements
ODRI's	Orthopaedic-relative infections
PCR	Polimerase chain reaction
rpm	Revolution per minute
SccMec	Staphylococcal cassette chromosome *mec*
SE	*Staphylococcus epidermidis*
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
X-Gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

LIST OF FIGURES

LIST OF PICTURES

LIST OF TABLES

ÖZET

KOAGULAZ NEGATİF STAFİLOKOKLARDAN BETA HEMOLİZİN GENİ'NİN KLONLANMASI VE BİYOLOJİK AKTİVİTESİNİN GÖSTERİLMESİ

Sahd A. Aydın Adnan Menderes Üniversitesi Sağlık Bilimleri Enstitüsü Moleküler Biyoteknoloji Anabilim Dalı (Disiplinlerarası), Yüksek Lisans Tezi, Aydın, 2021.

Koagülaz negatif stafilokoklar (KNS) serbest plazma koagülaz üretememelerinden dolayı yakından ilişkili oldukları fakat onlardan daha virulent olan *S. aureus*'dan ayrılmaktadırlar. KNS'lar, damar içi araç enfeksiyonlarından kaynaklanan en yaygın bakteriyemi sebebidir. Günümüzde önemli türler olan *S. epidermidis* ve *S. haemolyticus* klinik mikrobiyoloji laboratuvarlarında hastane kaynaklı ana patojenleri temsil etmektedirler.

Beta Hemolizin (*hlb*), *S. epidermidis*'in patogeneziyle ilişkili önemli bir virülans molekülü olarak hizmet etmektedir. Bu sitotoksin iki ayrı aksiyon mekanizmasına sahiptir. Bunlar: sfingomiyelinaz aktivitesi ve DNA biyofilm ligaz aktivitesi yani β-toksinin eksogenoz DNA varlığında kendisini çapraz bağlama yeteneğidir.

Bu çalışmada, Beta Hemolizin (*hlb*) proteinin aktivitesini ele almak için diyabetik ayak enfeksiyonlarından (DFI) alınan örneklerden *S. epidermidis* suşları kullanılmıştır. hlb genini çoğaltmak için *KpnI* ve *EcoRI* restriksiyon bölgeleri eklenmiş modifiye primerler dizayn edilmiştir. Çoğaltılmış gen vektör olarak *pUC19* plazmidi kullanılarak klonlanmış ve sekans analizine gönderilmiştir. Sekans sonuçlarına göre klonlandığımız gen, (*Staphylococcus epidermidis* strain Q47 sphingomyeline phosphodiesterase) genine 98.95% benzerlik göstermiştir. Kanlı agarda hemoliz testi yapılarak rekombinant protein varlığı doğrulanmıştır.

Anahtar Kelimeler: Beta hemolizin (*hlb*), Koagülaz negatif stafilokoklar (KNS), rekombinant gen, sfingomyelinaz, sitotoksin.

ABSTRACT

CLONING OF BETA HEMOLYSIN GENE FROM COAGULASE NEGATİVE STAPHYLOCOCCI AND TESTING OF BIOLOGICAL ACTIVITY

Sahd A. Aydin Adnan Menderes University Health Sciences Institute, Molecular Biotechnology (Interdisciplinary) Program, Master's Thesis, Aydin, 2021.

Coagulase-negative staphylococci (CoNS) are differentiated from the closely related but more virulent *Staphylococcus aureus* by their inability to produce free plasma coagulase. Coagulase-negative staphylococci are common etiologies of bacteremia that are resulted from intravascular device infections. They now represent one of the major nosocomial pathogens in clinical microbiology laboratories with *S. epidermidis* and *S. haemolyticus* being the significant species.

Beta hemolysin (*hlb*) serves as an important virulence molecule involved in the pathogenesis of *S. epidermidis*. This cytotoxin has two distinct mechanisms of action; Sphingomyelinase activity and DNA bio-film ligase activity (i.e. the ability of β-toxin to cross-link itself in the presence of exogenous DNA).

In this study, strains of *S. epidermidis* cultured from foot infections from diabetic patients were used. Primers with attached *KpnI* and *EcoRI* restriction sites were designed to amplify the *hlb* gene. The gene was subjected to cloning using pUC19 plasmid as a vector. After cloning, the amplicon obtained by M13 PCR was sent for confirmation by sequencing. From the sequence result, a percentage similarity rate of 98.95% of (*Staphylococcus epidermidis* strain Q47 sphingomyeline phosphodiesterase) was confirmed as expected. The recombinant protein was tested on a sheep blood agar which showed hemolysis of erythrocytes.

Keywords: Beta hemolysin (*hlb*), Coagulase-negative staphylococci (CONs), cytotoxin, recombinant gene, sphingomyelinase.

1. INTRODUCTION

Hospital-acquired infections are implications acquired from health care facilities, and typically occur in patients hospitalized for more than 48 hours. The advancement in medical knowledge and procedure in the field of medicine has enable the use of complex devices in the treatment of infections (Weinstein, 2001). These infections can be fatal to the host and acquired immunity, indicating the threat of these causative organisms (Wenzel, 2001).

Besides medical complications, there are high rate of morbidity and mortality especially among immunocompromised and elderly people. The continually increased in the high rate of antimicrobial resistance has lead to failed treatments, over time hospitalization and even deaths (ECDC, 2013; Cosgrove, 2003). Hospital acquired infections represents 5-10% of all the hospital admissions documented in the industralized countries, representing a critical condition for patients present in health care unit (Pittet et al., 2008 ; ECDC, 2013).

Antimicrobial resistance is a condition whereby microbes are resistance to antimcrobial drugs. This resistance creates a limitation on antibiotic therapy for the hard-to-treat multi-drug resistant bacterials. The reason behind this include; extensive consideration of resistance mechanisms, insufficient development of new drugs and moreover challenges from the regulatory requirements (Zaman et al., 2017).

ESKAPE pathogen is an acronym given to the group of bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) that constitutes in nosocomial infections (Rice, 2008). The European Center for Disease Prevention and Control (ECDC), reported that more than two million infections in the United States are caused by antibiotic resistant microorganisms per year, causing at least 23,000 deaths. This annual figure is projected to rise tenfold globally by 2050 (O'Neill, 2019).

Among the most clinical threatening species are the Staphylococcal species, which generally through the microscope appear as single, pairs and tetrads, or much more habitually as ''grape-like'' clusters. Staphylococci dwell as a non-spore forming, non-motile and facultative anaerobes. They are able to survive on natural surfaces and are broadly disseminated in nature (Bannerman, 2004).

Certain bacteria in the Staphylococcus genus are major human bacterial pathogens that causes various clinical manifestations (Lowy, 1998). They are usually found in the environment or as part of the human flora of healthy people (most frequently the nasal area). Some species of staphylococcus usually do not cause skin infections, however, their reach to the bloodstream or internal tissue causes variety of severe implications (Rasigade and Vandenesch, 2014).

Other staphylococcal species may cause opportunistic infections to humans and animals when they gain access to different body sites through damaged skin. Skin infections due to staphylococci, especially *Staphylococcus aureus* include; folliculitis, abscess, toxic epidermal necrolysis, cellulitis and impetigo, which are contagious (Lowy, 1998).

Staphylococci can be classified into two classes based on coagulase enzyme activity; Coagulase-positive staphylococci (CoPS) and Coagulase-negative staphylococci (CoNS). CoPS are capable of coagulating blood plasma. Examples of Coagulase-positive staphylococcal species are *S. aureus, S. schleiferi subsps. coagulans, S. intermedius, S. hyicus* and *S. delphini* (Bien et al., 2011). The most fatal staphylococcal species is *S. aureus*, which produces several pathogenic virulence factors enabling it to adhere to surfaces, damage tissues and escaping the immune system (Bien et al., 2011).

CoNS on the other hand, do not synthesize coagulase and they include species such *as S. epidermidis* and *S. haemolyticus* (Kloos, 1995). Coagulase-negative staphylococci consist of approximately 53 species. They are the most recognized among clinical isolates that are frequently identified during medication (Fey and Olson, 2010). CoNS are commensal on various flora of the body including the epidermal and mucosae. As a matter of fact, they display no tropism for different niches, but can be easily transferred from one person to another by touch or skin sloughing (Otto, 2009).

Due to the low virulence factors present in Coagulase-negative Staphylococci, they are considered as a simple commensal bacterium. However, the progress advancement of nosocomial CoNS infections has endowed scientists and technicians to rethink the status of these bacteria by making constant enquiries on its pathogenicity (Becker et al., 2014). The number of publications on CoNS genome has provided essential knowledge in understanding the pathogenicity of this bacteria. Virulence factors such as bio-film and adhesive proteins, has made CoNS to remain peculiar and contentious. CoNS appears to have a very broad gene repertoire encoding factors for adhesions, biofilm growth, hemolysins, exoenzymes and superantigens (Otto, 2009).

Hemolysins are identified as an essential virulence factors that play an important role in bacterial invasion and escaping from the host immune response (Salyers and Whitt, 1994). Staphylococcal hemolysins are classified into alpha hemolysin (α), beta hemolysin (β), gamma hemolysin (γ) and delta hemolysin (δ) (Bemheimer et al., 1974).

Beta hemolysin (*hlb*) gene is located on the 4 Kb *ClaI* (*Caryophanon latum I* site specific restriction endonuclease). It consists of 330 amino acids with a predicted molecular weight of 39 Kd. Beta-toxins do not form pores in the plasma membrane but rather, hydrolyses the lipid in the plasma membrane and phosphorylcholine which forms the sphingomyeline (Sireesha, 2019). The main objectives of this study;

1. Amplify beta hemolysin gene (*hlb*) among coagulase-negative staphylococci isolates by Polymerase Chain Reaction (PCR) using designed primers with restriction regions.
2. Cloning of the gene into *E. coli* (DH10B) using pUC19 plasmid vector.
3. To observe the hemolytic activity of the recombinant protein on erythrocytes using sheep blood agar.
4. Observing the hot-cold hemolysis exhibited by the recombinant *hlb* protein.

2. LITERATURE REVIEW

2.1. Classification of Coagulase-negative staphylococci (CoNS)

Since the early begining of Bacteriology, the research and classification of microorganisms that meet the current criteria of coagulase-negative staphylococci is been studied (Rosenbach, 1884). The first report on *Staphylococcus albus* was made by the German surgeon Friedrich J. Rosenbach in 1884. Somewhere later in 1891, *S. epidermidis* was discovered as a colonising germ of aseptic wounds (Welch, 1891).

However, later in the 1940's, the clarification of the word ''coagulase-negative staphylococci'' was postulated by the British Bacteriologist Ronald W. Fairbrother, who suggested the production of coagulase as a central theory in the grouping of staphylococci (Fairbrother, 1940). Till the late 1970's, the known CoNS were restricted to just two species; *S. epidermidis* and *S. saprophyticus* (Becker et al., 2014).

The classification system of CoNS was expanded by Kloos and Schleifer in 1975, by adding additional species to the already recognized *S. epidermidis* and *S. saprophyticus*. The classification was made by dividing coagulase-negative staphylococci into two classes, those indigenous to human and the non-human pathogens (Boyce, 1997). Species and sub-species of the heterogenous group of CoNS are identified in 2020, spanning to 53 species as shown in (**Table 1**). It is not difficult to forecast that due to the adoption of the principle of ''one health'', the increasing rise in surroundings and animal-related biocenosis will continue increasing the number of Staphylococcal organisms, in particular CoNS (Rock et al., 2009).

Table 1. Overview of the validly described Coagulase-negative staphylococcal species (Becker et al., 2020).

Species and sub species	***Effective species description***	***Main sources***
S. epidermidis	*Winslow and Winslow 1908; Evans 1916*	Human and animal (e.g. cat, cattle, dog, goat, gorilla, horse, pig, sheep) skin; fermented food
S. saprophyticus	*Fairbrother 1940; Shaw et al. 1951*	
subsp. saprophyticus	*Fairbrother 1940; Hájek et al. 1996*	Human and animal (e.g. cat, goat, horse, pig, sheep) skin
subsp. bovis	*Hájek et al. 1996*	Cattle skin
S. cohnii	*Schleifer and Kloos 1975*	
subsp. cohnii	*Schleifer and Kloos 1975; Kloos and Wolfshohl 1991*	Human and animal (e.g. cat, dog, goat, pig, poultry) skin
subsp. urealyticus	*Kloos and Wolfshohl 1991*	Human and animal (e.g. apes, monkeys, horse) skin
S. haemolyticus	*Schleifer and Kloos 1975*	Human and animal (e.g. cat, cattle, dog, horse, goat, pig, sheep) skin; fermented food, milk

S. xylosus	*Schleifer and Kloos 1975*	Human (rare) and animal (e.g. cat, goat, horse, insectivores, primates, rodents, sheep) skin; fermented food
S. warneri	*Kloos and Schleifer 1975*	Human and animal (cat, dog, goat, horse, insectivores,monkeys, pig, prosimians, rodents, sheep) skin; fermented food
S. capitis	*Kloos and Schleifer 1975*	
subsp. capitis	*Kloos and Schleifer 1975; Bannerman and Kloos 1991*	Human and animal (e.g. cat, dog, horse) skin
subsp. urealyticus	*Bannerman and Kloos 1991*	Human skin
S. hominis	*Kloos and Schleifer 1975*	
subsp. hominis	*Kloos and Schleifer 1975; Kloos et al. 1998*	Human and animal (e.g.cat, dog, goat, pig, sheep) skin
subsp. novobiosepticus	*Kloos et al. 1998*	Unknown
S. simulans	*Kloos and Schleifer 1975*	Human and animal (e.g. cattle, horse, sheep) skin
S. sciuri	*Kloos et al. 1976; Švec et al. 2016 (rejection of former subspecies classification8)*	Human and animal (e.g. cat, cattle, dog and other carnivores, dolphins, goat, horse, insectivores, marsupials, monkeys, pig,rodents, turtles, whales) skin

S. lentus	*Kloos et al. 1976; Schleifer et al. 1983*	Animal (cat, cattle, goat, horse, mink, pig, poultry, sheep) skin; food (clams, meat, milk, soy bean oil meal)
S. chromogenes	*Devriese et al. 1978; Hájek et al. 1987*	Animal (cattle, pig, horse, goat, sheep) skin; food (milk)
S. saccharolyticus	*Kilpper-Bälz and Schleifer 1984 (effective publication 1981)*	Human and animal (gorilla) skin; contaminated platelet Concentrates
S. carnosus	*Schleifer and Fischer 1982*	
subsp. carnosus	*Schleifer and Fischer 1982; Probst et al. 1998*	Fermented food and starter cultures, animal (cattle) skin
subsp. utilis	*Probst et al. 1998*	Fermented food
S. auricularis	*Kloos and Schleifer 1983*	Human external auditory canal as principle habitat, rarely from other places of the human and from animal (cattle) skin
S. gallinarum	*Devriese et al. 1983*	Animal (chicken, pheasant) skin
S. caprae	*Devriese et al. 1983*	Animal (cat, goat) skin
S. arlettae	*Schleifer et al. 1984 (effective publication 1985)*	Animal (e.g. cattle, goat, pig, poultry, sheep) skin; environmental samples

S. equorum	*Schleifer et al. 1985*	
subsp. equorum	*Schleifer et al. 1985; Place et al. 2003*	Animal (e.g. cattle, goat, horse, sheep) skin; fermented food
subsp. linens	*Place et al. 2003*	Food (cheese starter culture)
S. kloosii	*Schleifer et al. 1984*	Animal (goat, pig) skin
S. lugdunensis	*Freney et al. 1988*	Human and animal (e.g. cat, cattle, chinchilla, dog, goat, guinea pig) skin
S. schleiferi	*Freney et al. 1988*	
subsp. schleiferi	*Freney et al. 1988; Igimi et al. 1990*	Human and animal (e.g. cat, dog) skin
S. felis	*Igimi et al. 1989*	Animal (cat, horse) skin
S. piscifermentans	*Tanasupawat et al. 1992*	Fermented food and starter cultures
S. pasteuri	*Chesneau et al. 1993*	Fermented food; animal (pig) skin
S. succinus	*Lambert et al. 1998*	
subsp. succinus	*(Lambert et al. 1998) Place et al. 2003*	Animal (cat, cattle, insectivores, rodents, songbirds) skin; fermented food and starter cultures
subsp. casei	*Place et al. 2003*	Animal (insectivores, rodents) skin; fermented food
S. condimenti	*Probst et al. 1998*	Fermented food and starter cultures
S. fleurettii	*Vernozy-Rozand et al. 2000*	Animal (goat, pig, small mammals) skin; food (milk, cheese)

S. pettenkoferi	*Trülzsch et al. 2007 (effective publication 2002)*	Human and animal (cat) skin
S. nepalensis	*Spergser et al. 2003*	Animal (cat, goat, pig, squirrel, monkeys)
S. massiliensis	*Al Masalma et al. 2010*	Human skin
S. stepanovicii	*Hauschild et al. 2010 (effective publication 2012)*	Small mammals` (insectivores, rodents) skin
S. microti	*Nováková et al. 2010*	Mice skin; bovine milk
S. rostri	*Riesen and Perreten 2010*	Animal (pig, water buffalo) skin
S. devriesei	*Supré et al. 2010*	Cattle skin, food (milk, cheese
S. petrasii		
subsp. petrasii	*Pantůček et al. 2013*	Human skin
subsp. croceilyticus	*Pantůček et al. 2013*	Human ear canal reports
subsp. jettensis	*De Bel et al. 2013, De Bel et al. 2014*	Unknown (possibly human skin)
subsp. pragensis	*Švec et al. 2015*	Unknown (possibly human skin)
S. argensis	*Hess and Gallert 2015*	Unknown (one isolate from river sediment)
S. edaphicus	*Pantůček et al. 2018*	Unknown (two environmental isolates)
S. caeli	*MacFadyen et al. 2019*	Unknown (one environmental isolate)

S. pseudoxylosus	*MacFadyen et al. 2019*	Unknown (one isolate from bovine mastitis)
S. debuckii	*Naushad et al. 2019*	Unknown (four isolates from bovine milk)

2.2. Staphylococcal virulence factors

Virulence factors are molecules produced by pathogenic organisms that determine their ability to infect or damage host cells or tissues. They play a massive role in the mediation of immune suppression, invasion and weakening of host cells (Cross, 2008). *S. aureus* devotes a significant amount of potential molecules to equivocate the host immune system (Kusch, 2014).

2.2.1. Toxins

Staphylococcal pathogenecity is distinguished by the production of it's potential virulence factors (Otto, 2010; Bartlett et al., 2010). Among the virulence factors secreted are toxins; which play an essential role in infections. Staphylococcal toxins are categorised into two major classes; cytotoxins and superantigens based on their lytic activity on cells.

Cytotoxins produces lesions directly to specific outer membranes of cells. The superantigenic toxins on the other hand, do not directly produce lytic action but can produce lesions via the over secretion of cytotoxins derived from activated T-cells and monocytes/macrophages (Cunha et al., 2008).

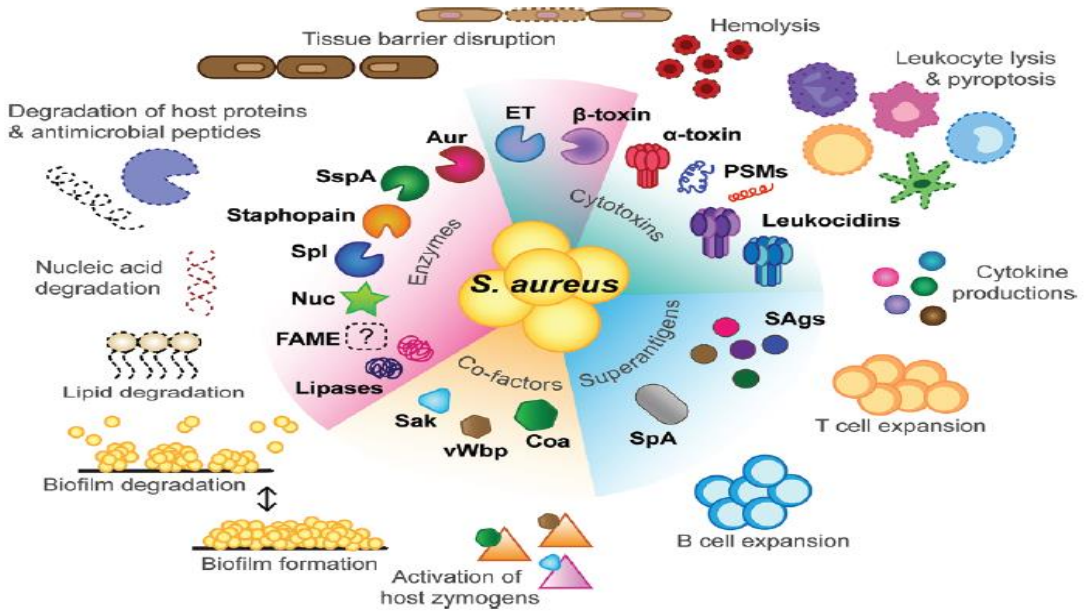


Figure 1. Virulence factors secreted by *S. aureus* (Tam and Torres, 2019).

2.2.2. Leukotoxin

It is proposed that Staphylococcal leukotoxins induce multiple cell responses that may be linked to the activations of Ca^{2+}. The pore-forming leukotoxins produces two seperate protein components, ‘‘S’’ and ‘‘F’’, which together form β- barrel pores (Wang et al., 1990).

The S-component confers specificity of the cell type by binding to the cellular receptors. A conformational shift allows the F-component dimerisation to be induced when combined to the cell receptors to form a pre-pore which the the β-barrel transmembrane channel is inserted (Yamashita et al., 2011). Leukotoxin are generally involved in cell activation, and might also aid in the induction of apoptosis. Leukotoxins at doses below 1nM stimulate the activation of the inflamoasome, a cytolitic multiprotein oligomer (Perret et al., 2012).

2.2.3. Panton-valentine leukocidin

Panton-valentine leukocidins (PVL) is a member of the pore-forming cytotoxin which attacks mononuclear cells of humans and rabbits. Most researches have shown the synergistic activity of the exoproteins; LukS-PV and LuKF-PV, which contributes the toxic effectivity of panton-valentine leukocidin (Yoong et al., 2013).

The LukS-PV binds to the supplementary receptor; C5aR, a hetero-oligomerization of the S part with F segment which brings about the inclusion of a hydrophobic stem into the layer of the target cell that traverses the host lipid bilayer (Spaan et al., 2017). The arrangement and formation of pores by PVL prompt cell lysis because of the spillage of divalent cations that are the foundational background for cell homeostasis. The main targets of PVL are polymorphonuclear leukocytes (PMNL’s, neutrofils), with high species specificity (Löfer and B.et al., 2010).

2.3. Hemolysins

2.3.1. Alpha Hemolysin (Hla-Toxin)

The analysis and examination related to toxic activity on staphylococcal supernatants was commenced in the early 1800's. The study of alpha toxin began after a calamity which took place in Bundaberg, Australia in 1928; 21 children vaccinated with diphtheria toxoid got sick and twelve children from the victims died (Sireesha et al., 2019).

Alpha-toxin is a hemolytic pore-forming toxin responsible for damaging several mammalian cell membranes. Alpha-hemolysin neurotoxic and dermonecrotic toxin can also be lethal in most animal species. The first cloning of alpha-hemolysin gene was performed from the chromosome of *S. aureus,* which was later sequenced by Gray and Kehoe in 1984 (Gray et al., 1984).

Alpha toxin consists of 33 KDa polypeptide and are generally produced by 95% of clinical *S. aureus* strains (Honeyman, 2006; Grumann et al., 2014). Alpha toxin is a beta-barrel forming toxins secreted as a water-soluble monomer (Otto, 2014). The toxin alone is not necessarily known to be toxic, but its binding ability and formation of oligomerization into heptameric structure on the host cell layer makes it life-threathening (Yamashita et al., 2011). Alpha toxin produces a complete hemolytic zone on blood agar, exhibiting lytic effects on a variety of mammalian erythrocytes, like rabbit, ox, sheep (Eugenio, 2018).

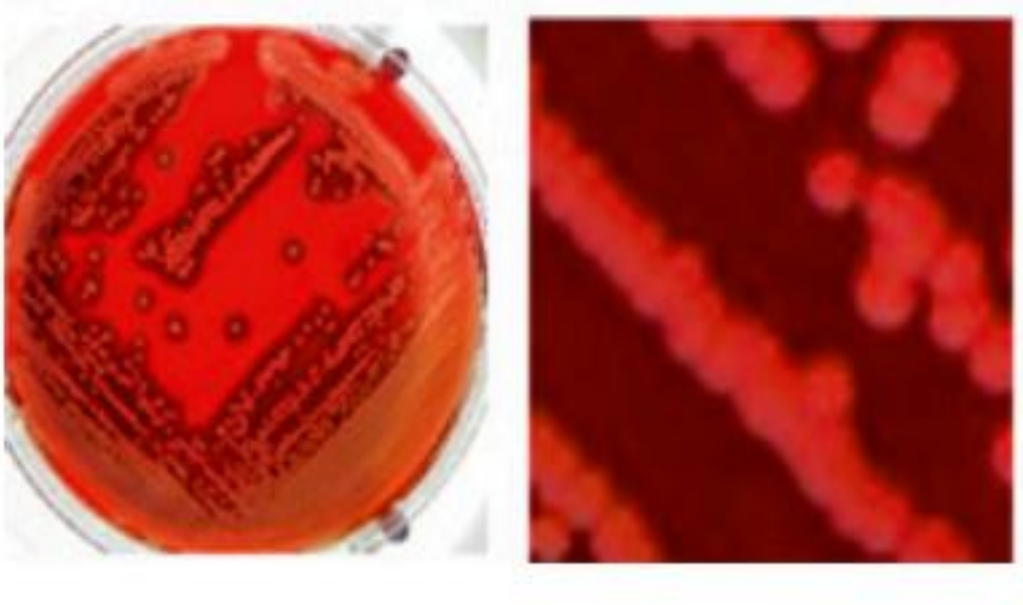


Figure 2. Activity of Alpha toxin on sheep blood agar (Lukas et al., 2014).

2.3.2. Beta hemolysin (β- toxin)

Initially classified as a non-porous hemolysin, beta hemolysin was first identified and recognised by Glenny and Stevens in 1935. Later in 1989, the sequence of *hlb* gene was discovered after several unique observations were analyzed (Glenny et al., 2018).

Beta hemolysin is a Mg^{2+} dependent sphingomyelinase C. Sphingomylinases are phosphoric diesters which cleave abundant sphingomyelin in eukaryotic membrane. Beta toxin is capable of degrading sphingomyelin present in the outer membrane of erythrocytes phospholipid layer, producing ceramide and phosphorylcholine. Most studies reveal that Beta hemolysin has an isoelectric point of 9.0 which lyses erythrocytes (Flores-Diaz et al., 2016).

Encoding gene of this toxin is entirely chromosomal. Beta hemolysin consists of 330 amino acids with a molecular weight of 39 Kd. Moreover, there is a deletion of signal peptide which is identified by the first 34 amino acids during secretion (Sireesha et al., 2019). The exact role of this toxin in the pathogenecity of staphylococcal bacteria was not known in the previous years. Recent studies however, have now postulated the understanding of beta hemolysin, due to it's effective activity on animal isolates (Dinges et al., 2000).

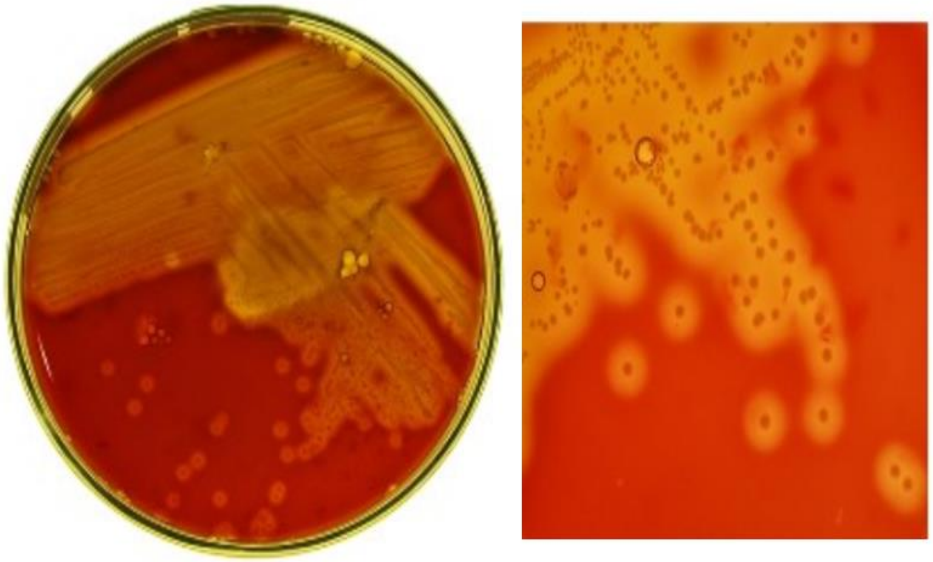


Figure 3. Beta toxin activity on blood agar (Niederstebruch et al., 2017).

2.3.2.1. Hot-cold phenomenon

Beta hemolysin is a sphingomyelinase with hot-cold ability. This is due to the hemolytic activity of the toxin after a subsequent reincubation below 10℃ after treatment at 37℃. Much has been written about the mechanism of this reaction, but it's mechanism was not fully understood according to the literature from 1963 (Doery, 1965).

Wiseman postulated that pH or NaCl concentrations in the erythrocytes suspensions treated with hemolysin at 37℃ are rapidly altered. The author also suggested that decrease in temperature is an essential factor since it may break weak bonds and cause weakening of the membranes (Medora et al., 2007).

Meduski and Hochstein (1974), further observed the "hot-cold" stage resulting in changes in the choline residues of the sphingomyelin membrane. The Lytic effect of I_3^- on erythrocytes were compared to those found in ‘‘hot-cold’’ lysis (Meduski, 1972). It was also discovered that at pH 7.0, I_3^- are capable of interacting with $-N^+(CH3)_3$ groups of other phospholipids, leading to "hot-cold" lysis. Beta toxin is highly sensitive to the erythrocytes of sheep, cow and goats, whereas it expresses an intermediate sensitivity in human and rabbit, with highly resistance expressed in murine and canine erythrocytes (Larsen et al., 2002).

2.3.2.2. Mode of action of beta hemolysin

The framework of beta toxin is identical to the sphingomylinases synthesized by *Listeria ivanovii*, *Leptospira interrogans* and *Bacillus cereus* (Goni, 2002). The sphingomyelinase activity including lymphotoxicity and hemolysis enable the biological activity of beta toxin. Based on the structure, it is suggested that beta toxin is capable of DNA cleavage and it's classified into the superfamily of DNase I (Husebuy, 2007).

Beta toxin is a Mg^{2+} dependent neutral sphingomyelinase which breaks down sphingomyelin into phosphorylcholine and ceramide [N-acyl sphingosine]. Ceramide products play an essential role in the cells of eukaryotic organisms like stimulation of nitrogen-activated protein kinases, activation of second messenger system, modification of cell morphology and even apoptosis (Futerman, 2004). Beta hemolysin damages the human monocytes, polymorphonuclear leukocytes, keratinocytes and lymphocytes. Despite the indirect cell lysis of beta toxin on cell, it leads cells to be vulnerable to the other toxins such as phenol-soluble modulins (PSMs) (Cheung, 2012). Infections caused by Beta toxin produces enormous lesions in the tissues of organisms. Nevertherless, it does not necessarily cause damage in the endocarditis of rabbit and pneumonia models (Salgado, 2014).

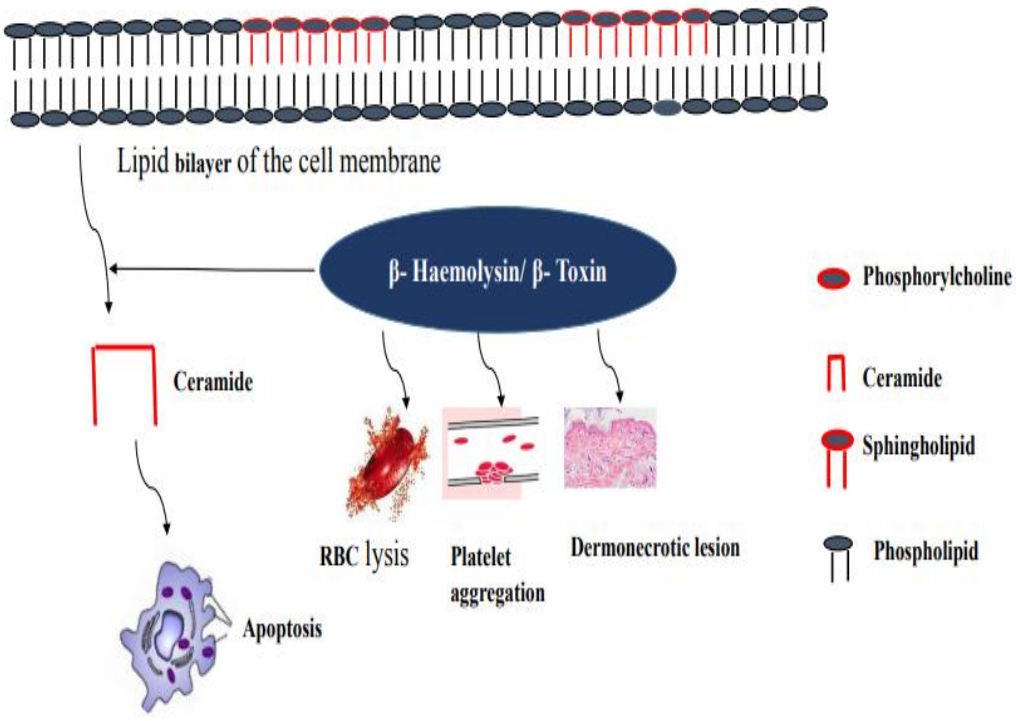


Figure 4. β-toxin activity on the cell membrane (Sireesha et al., 2019).

2.4. Gamma hemolysin (γ- toxin)

In 1938, gamma toxin was discovered by Smith and Price. After consecutive researches made, it was discovered that gamma hemolysin was more sensitive to rabbit RBCs and also more resistant to the RBC of fowl. The gene ecoding γ- hemolysin is generally found in *S. aureus* isolates related to humans (Sireesha et al., 2019).

Gamma hemolysin consist of two-components of exotoxin which comprises of six different combination of proteins which are active against erythrocytes (Dinges, 2000). The toxin is recognized by an ion-exchange column elution comprising of two non-associated and water soluble peptides in the ‘‘S’’ and ‘‘F’’ segment (Gravet, 1998; Ventura, 2010). This gene is occupied within a 4.5 Kb *ScaI* fragment of *S. aureus* chromosome. The hemolysin gene exerts cytolytic activity, especially on neutrofils (action at 10 ng/mL) and microphages on different types of mammalian erythrocytes (Sireesha et al., 2019).

2.5. Delta hemolysin (δ-toxin)

Williams and Harper, detected delta hemolysin for the first time from it’s activity on sheep blood agar plates in 1947. Delta hemolysin is composed of 26 amino-acids with α-helix structure, which act as a surfactant in the disintegration of cell membrane. Disruption of the cell membrane results in membrane damage in a variety of mammalian cells, as well as membrane-bound organelles (Sireesha et al., 2019).

In one of the studies, after the addition of an anti-α and anti-β hemolysins, observation was confirmed after displaying a narrow zone of complete hemolysis on the sheep blood agar (Hébert, 1985). Molecular weight of delta protein is approximately 30 Kd. Several activities and hemolytic action of this protein have been attributed to delta hemolysin (Sireesha et al., 2019).

2.6. Genome based analysis of *Staphylococcus epidermidis* chromosome

The genome-based analysis of virulence genes among various pathogenic bacterials has been executed in various dimensions. The first genome-based analysis on CoNS was observed in a non-biofilm, non-infection associated *S. epidermidis* strain (ATCC 12228) which was detected in an antibiotic residual food product.

The second study was performed on *S. epidermidis* with reference code RP62A (ATCC 35984); a slime-producing and methicillin-resistant biofilm isolate (Raue et al., 2020). Based on the studies, the genome of *S. epidermidis* ATCC 12228 strain consists of a 2 499 279 bp and six plasmids. Some of the pathogenic virulence factors attained in the *S. epidermidis* ATCC 12228 genome are listed in the (**Figure 5**) below;

Adhesins	ORF	name	Function
Autolysin	SE1881	atlE	Adhere to polymers (Heilmann *et al.*, 1997)
Fibrinogen binding protein	SE0331	sdrG(fbe)	Fibrinogen binding protein (Nilsson *et al.*, 1998;Davis *et al.*, 2001)
SD-rich cell surface adhesin	SE2395, SE1632	sdrF,sdrH	Not known (McCrea *et al.*, 2000)
Extracellular matrix binding protein Embp	SE1128	embp	Fibronectin binding protein (Williams *et al.*, 2002)
Elastin-binding protein	SE1169	ebpS	Adhesion on host proteins (Park *et al.*, 1996)
Cell accumulation	SE0175	aap	Accumulation associated protein (Accession No: AJ249487)
Fmt	SE0754	fmt	Autolysis and penicillin resistant (Komatsuzawa *et al.*,1997)
Exoenzymes			
Serine protease, V8 protease	SE1543	sspA	Degrade or digest proteins (Rice *et al.*, 2001)
Glycerol ester hydrolase	SE2403	geh	Degrade lipids (Simons *et al.*, 1998)
Lipase A	SE0424		Possible degrade lipids
Lysophospholipase	SE0980		Possible degrade lipids
Putative esterase lipase^{-1}	SE0389		Possible digest lipids
Putative carboxyl esterase	SE2328		Possible degrade lipids
Metallosprotease	SE2219	SepP1	Elastase (Teufel and Gotz, 1993)
Protease ClpX	SE1349	clpX	Degrade or digest proteins
Thermonuclease	SE1004	nuc	Digest host nucleic acids
Putative 5′-3′exonuclease	SE1130		Possible degrade host nucleic acid
Exonuclesae	SE1028, SE1029		Possible degrade host nucleic acid
Toxins			
Delta-haemolysin	SE1634	hld	Destruction of blood and tissue cells (McKevitt *et al.*,1990)
Beta-haemolysin	SE0008		Phospholipase C synthesis (Katerov *et al.*, 1994)
Others			
67 KDa myosin cross-reactive protein	SE0776		Cross-reactive with host cardiac myosin (Dale andBeachey, 1985)
Putative lipoprotein similar to streptococcal PsaA	SE0405		Putative adhesin essential for virulence (Berry andPaton, 1996)
Chitinase B	SE0760		Invasion of skin
Putative protein similar to attachment and virulence	SE1951		Possible host cell attachment
Similar to streptococcal haemagglutinin	SE2249		Unknown
Lactococcal lipoprotein	SE2320		Possible host cell attachment

Figure 5. Pathogenic virulence factors detected in *S. epidermidis* ATCC 12228 strain (Zhang et al., 2003).

2.7. Pathogenecity of *Staphylococcus epidermidis*

S. epidermidis is the most frequently isolated species from human skin epithelial, axillae (armpits), head and nares (nostrils) (Kloss, 1986). Based on clinical documented reports, *S. epidermidis* is just second to *S. aureus* as the most common species encountered in Orthopaedic Device-Related Infections (ODRIs) (Trampuz, 2005).

It is proposed that *S. epidermidis* may have a probiotic function by preventing colonization of the host from other serious pathogens, such as the *S. aureus* (Lina, 2003). Much studies have been observed on the mechanism by which *S. epidermidis* encourge clinical infections among coagulase-negative staphylococci (Uckay, 2009; Rogers, 2009).

S. epidermidis represents one of the most identified agents of infections relating to indwelling medical devices, such as peripheral or central intravenous catheters (CVCs). These infections typically start with the introduction of bacteria from the skin of a healthy patient or from the health staff during device insertion (O'Grady, 2002).

Due to the minimum virulence potential of *S. epidermidis*, it is endowed with determinants that promote enduarance like molecule for immune invasion, rather than toxins to attack the host (Faurschou, 2003).

2.7.1. Bio-film formation of *Staphylococcus epidermidis*

The ability to bind to the surface is the first step in the bio-films formation of bacterials. Biofilms are multicellular, surface-attached agglomerations of microorganisms. Biofilm formation proceeds after the initial adhesion of cells to surfaces and their subsequent aggregation into multicellular structures (Otto, 2009). Adhesion forces of the the cell-to-cell colonization and surfaces interaction is required for the composition of biofilm. Medical and surgical treatment procedures are often complicated as a result of bio-film. This is because implant removal is required to remove bio-film (O'Toole, 2000).

There are three dynamic stages that are involved in biofilm formation; (I) initial binding of planktonic cells, (II) assemble into numerous bacterial layers and maturation of the biofilm structure and the final stage (III) seperation and dispersion of planktonic cells (Costerton et al., 1999; Sutherland, 2001; Flemming, 2010).

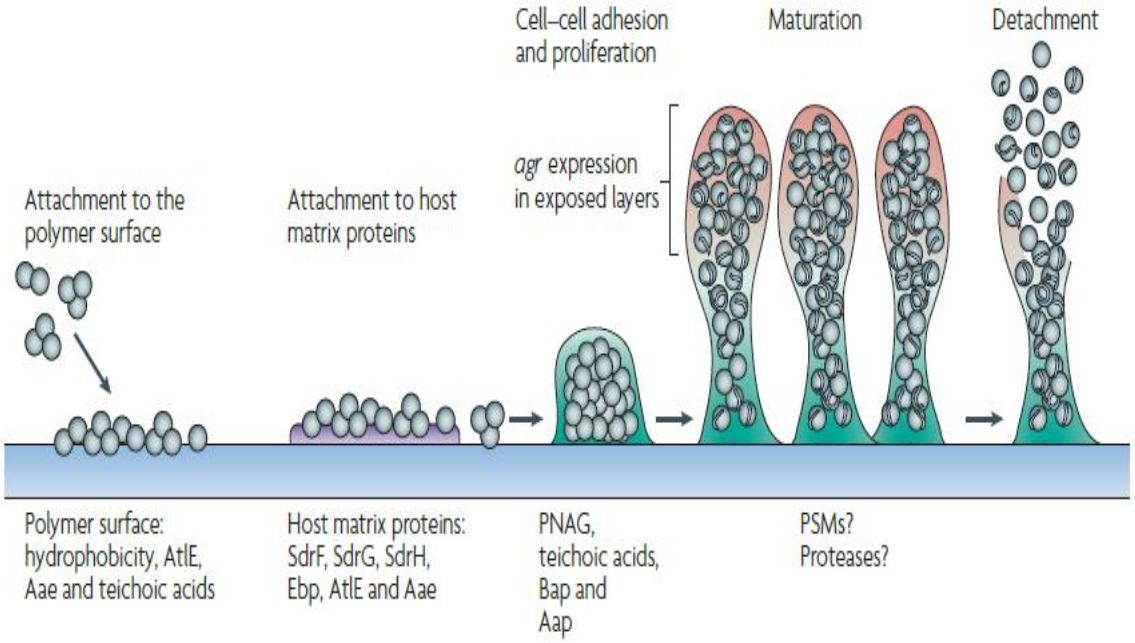


Figure 6. Bio-film formation of *S. epidermidis* in relation to the virulence factors in each phase (Otto, 2009).

2.7.2. *Staphylococcus epidermidis* adhesive activity

S. epidermidis is a commensal microorganism that retains the ability to specifically adhere to host proteins within the skin layer. Specific staphylococci cell walls proteins that binds to the extracellular matrix molecules (such as the fibronogen and collagen) have been identified (Wilkinson, 1997).

Bacterial initial adhesion to implant surfaces is intervening by a non-specific interaction such as hydrophobic interactions and specific adhesins like autolysin (*AtlE*), extracellular DNA (eDNA) and staphylococcal surface protein 1 and 2 (SSP-1, SSP-2) (Izano, 2008). The presence of the vast array of surface proteins; MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) enable *S. epidermidis* to interact with matrix proteins during protein adhesions (Brennan, 2009).

Virulence factor	*Gene*	*Function*
AtlE	*atlE*	*An abundant bi-functional autolysin and adhesin that affects surface hydrophobicity*
Aae	*aae*	*A bi-functional autolysin and adhesin*
Teichoic acids	*Multiple biosynthetic genes*	*In Staphylococcus aureus, teichoic acids affect attachment through the binding of autolysin*
SdrF	*sdrF*	*Binds to collagen*
SdrG (also known as Fbe)	*sdeG (fbe)*	*Binds to fibrinogen*
SdrH	*sdrH*	*Putative binding function only*
Ebp	*Ebp*	*Binds to elastin*
AtlE and Aae	*atlE and aae*	*Binds to various matrix proteins*

Figure 7. Bio-film formation genes of *Staphylococcus epidermidis* (Otto, 2009).

2.7.3. Phenol-Soluble Modulins (PSMs)

Phenol-soluble modulins were discovered in the 1999 after an examination was made on *S. epidermidis.* During the studies, three peptides were identified; PSMα, PSMβ and PSMγ which were characterized as an inflammatory complex extracted from the culture filtrate by hot phenol principle (Mehlin, 1999).

It was thought until recently, that there were no toxins produced by *S. epidermidis*. However, the identification of PSM's has now altered the notion. Phenol soluble modulins are family of the genome-encoded peptides regulated by the *agr* quorum sensing system (Wang, 2007).

2.7.4. Antibiotic Resistance of *Staphylococcus epidermidis*

Another essential virulence factor that helps in the pathogenecity of *S. epidermidis* is its resistance to antimicrobial agents, which can be acquired or innate (Tenover, 2006; Mah, 2001). Most *S. epidermidis* strains remain susceptible to the newer produced antibiotics such as (daptomycin, tigecycline, linezolid and dalbavancin) in clinical manifestations (Pinheiro, 2016). The high endemic antimicrobial resistance within this species represents a significant challenge in the treatment of *S. epidermidis* infections. Several nations, including the United States, have accounted that between 75-90% of all the hospital isolates of *S. epidermidis* are resistance to methicillin, a first choice antibiotic against Staphylococcal infections. This frequency is even higher in correlation than the corresponding rate for *S. aureus* (40-60%) (Diekema, 2001).

Resistance to methicillin is encoded on a mobile genetic elements (MGEs), Staphylococal cassette chromosome *mec* (Scc*mec*). This cassette chromosome contains the *mecA* gene, which encodes a penicillin-binding protein, PBP2a, with decrease affinity for methicillin compared with the affinities of other PBPs (Ma, 2002). Ten different SCC*mec* structures are identified in *S. epidermidis*. The most abundant form was SCC*mec* type IV, which appears to have the shortest structure (Miragaia, 2005). *S. epidermidis* strains have constantly become resistance to several other antibiotics, including rifamycin, fluoroquinolones, gentamycin, tetracycline, chloramphenicol and sulphonamides (Rogers, 2009).

2.7.5. Host immune response to *Staphylococcus epidermidis*

Adaptive immunity refers to the long lasting immune responds and antigen-specificity controlled by lymphocytes. This immunity can be broadly classified into two categories; cellular response and humoral response. The cellular response is represented by T helper (*Th*) and cytotoxic (*T*) lymphocytes. The humoral response constitutes B lymphocytes and antibodies. The role of infection clearance by adaptive immune response was highlighted in an in vivo model, using immunocompromised mice. The result obtained showed that mice lacking T cells or T and B cells were highly susceptibility to *S. epidermidis* (Vuong, 2008).

Evidence that the skin microbiota in general, have an important impact on the human immune system has been postulated. *S. epidermidis* interacts with the host immune system which contributes an essential role in the immunological tolerance (Belkaid, 2016). The migration of neutrophils to infected sites are empowered by the host signals (e.g. chemokines, AMPs). Phagocytosis by neutrophils is one of the most important mechanisms for eliminating infected bacteria from our system. At the infected site, neutrophils will internalize opsonized bacteria forming a phagosome and finally, the bacteria will be destroyed in the phagolysosome by the action of reactive oxygen species (ROS), proteases and antimicrobial proteases (AMPs) (Scharschmidt, 2015).

Studies have been performed to assess the immune response to CoNS bio-film associated infections with the most of the studies focused on *S. epidermidis*. Moreover, the host immune response to *S. epidermidis* biofilm-associated infection is not adequate to eliminate the infection, which make most *S. epidermidis* biofilm-associated infections to be chronic and persistence in nature (Rogers, 2009).

3. MATERIAL AND METHOD

3.1. Materials

3.1.1. Bacteria strains

Coagulase-negative Staphylococci strains used in this study were previously collected from Diabetic patients with foot infection from Aydın Adnan Menderes University Hospital. Stock cultures of the CoNS were preserved at 4^{o} C with glycerol (15% v/v) at REDPROM laboratory, Aydin Adnan Menderes University. From the samples taken, *Staphylococcus epidermidis* strains (*SE12, SE21, SE31, SE50, SE57, SE58* and *SE74*) were selected for further studies.

3.1.2. Media and Chemical solutions

Tryptic Soy Agar (TSA)

12.0 g TSA media was dissolved in 400 mL distilled water. The solution was mixed thoroughly by a magnetic stirrer and then autoclaved at 121^{0} C for 15 minutes. It was then poured into sterile petri dishes.

Tryptic Soy Broth (TSB)

A weighted 9.0 g of a ready TSB media was dissolved in 300 mL distilled water and then autoclaving at 121^{0} C for 15 minutes. It was poured into sterile petri dishes.

Blood Agar

Blood Agar is a general-purpose enriched medium used to distinguish bacteria based on their hemolytic properties. 3.0 g of Tryptic Soy Broth (TSB) powder and 1.5% Agar was weighted, dissolved in 100 mL distilled water and mixed thoroughly by a magnetic stirrer. It was then autoclaved at 121^0 C for 15 minutes. After cooling to 45^0 C, 5 mL of sterile sheep blood (Biomerieux, Ca. No. 55822) was aseptically added and mixed thoroughly. The mixture was poured into sterile Petri dishes.

AXI Agar

9.0 g of a ready TSA media was dissolved by heating in 300 mL distilled water and then autoclaved at 121^0C for 15 minutes. The media was allowed to cool to 45^0C -50^0C. A proportional volume of 1.5 mL IPTG, 0.75 mL X-Gal and 0.3 mL Ampicillin (50 mg/mL) was added and mixed thoroughly. It was poured into sterile Petri plates. This media was used for the Blue-white screening of bacteria colonies for detection of recombinant bacteria during cloning.

Phenol: Chloroform: Isoamyl alcohol

A proportion of 25 mL of Phenol, 24 mL of Chloroform and 1mL of Isoamylalcohol was well mixed together and stored at $+4^0$ C. It was used in the denaturing of proteins and facilitates the separation of aqueous and organic phases.

(TBE) 10X

A weighted mass of 108 g Tris Base, 55 g Boric acid and 9.3 g EDTA were mixed together. 1000 mL of deionized water was added and the pH was adjusted to 8.3 using concentrated HCl. The stock was stored at room temperature.

70% Ethanol

For a stock solution of 500 mL, 70% ethanol was prepared by mixing 350 mL of 100% ethanol with 150 mL of distilled water.

3M Sodium Acetate

24.6 g of sodium acetate (anhydrous) was weighed. 70 mL of MilliQ water was added and mixed together by a magnetic stirrer. The pH was adjusted to 5.2 by adding glacial acetic acid. The solution was used in the precipitation process.

Ampicillin (50 mg/mL) stock solution

0.5 g of sodium ampicillin was dissolved in 5 mL distilled water. With a sterile syringe filter, the decanted stock was collected and stored at -20^0C. This was used in the preparation of AXI media and during plasmid isolation.

Kanamycin (50 mg/mL) stock solution

0.5 g kanamycin was dissolved completely in a 10 mL sterile water. 5 mL of sterile water was drawn through the sterile syringe and the decanted stock was stored at -20^0 C.

3.2.Method

The immediately following sections will describe the procedures employed to generate the cloned target gene of interest and also testing of the biological activity of the recombinant protein on sheep blood agar.

3.2.1. Bacterial DNA isolation

The isolates were streaked aerobically on a Tryptic Soy Agar (TSA) and incubated at 37^0 C for 24 hours. DNA isolation was performed using the Lysis method as given below:

- A colony from the culture plate was taken with a sterile loop.
- The colony was transfered into eppendorf tubes containing 1 mL distilled water and stirred to homogenity.
- The ependorf tubes were centrifuge for 5 mins at 11000 rpm
- The supernatants were discarded
- 100 µL of Lysis solution were added and pippeted to homogenity
- The tubes were incubated at 56^0C for 30 mins and a subsequent reincubation at 100^0C for 10 mins
- The tubes were vortex and stored at -20^0 C until use.

3.2.2. PCR primer sequence design

The primer sequence was designed to amplify beta gene (*hlb*) of *S. epidermidis* RP62A (GenBank: CP000029). The sequence of the gene was 1005 bp as shown in (**Figure 8**) below;

```
   1 atgaaacgag gtgtaacaat attgaattgg caacgtaaat gtatactaac tactttgttg
  61 gttttaagta gtttattttt agtattttcg actatcacat atgcgagtga acgtgatttt
 121 aaagacagtc ttaaaatcac tacacacaac gtgtatttct tacctactgc tatctaccct
 181 aattggggac aatctcagcg cgctgattta atttcaaaag cagattacat tcaaaatcaa
 241 gatgtcgtga ttctaaatga attatttgat aaaaaagctt ctaaaagatt gttaacacgt
 301 ctacattcac agtaccccta tcaaacacct atcgttggta agggtacaga aggttggcaa
 361 aatacttctg gtacttatag aaaaattaaa aaagtaagtg gtggcgttgg tattgtgagt
 421 aaatggccta tcgtacaaca agaacaacat atttataaaa aaggttgtgg ggctgatatg
 481 gcaggtaata aaggctttgc ctacattaaa attaataaga atggcaaata ccaccatatt
 541 atcggaacac atctacaagc tgaagatcca acatgcttta aaggaaaaga taaggacatt
 601 agacagagtc aaatgagtga aattaaacag tttatcaaag acaagaatat ccctaaaaat
 661 gaacccgtct atatcggtgg tgacttaaat gtcattaaag attcagatga atatcaacaa
 721 atggcaaata acttaaatgt ttcattacct actcaattcg atggtaatgc atatagttgg
 781 gatactagca gtaatagtat tgcgaaatat aattatccta aattagaacc tcaacactta
 841 gattatattt tattagatcg tgaccatgca caaccaagct catggcataa tgatacacat
 901 agagtgaagt caccagaatg gtccgtgaaa tcttggggta aaacatacaa atacaatgat
 961 tactcagatc attacccact ctctggctat gcatcaaatg aataggacaa t
```

Figure 8. Sequence of Beta hemolysin gene of *S. epidermidis* RP62A (GenBank: CP000029).

Restriction enzyme sites for cloning at the 5′ and 3′ ends into the pUC19 vector were added. The primer sequence was designed and modified by adding *Kpn1* and *EcoR1* restriction sites using comparative tools such as NCBI, Reverse compliment, Multiple Primer Analyser and Nebcutter.

Table 2. Forward and Reverse primers with restriction enzyme regions.

Fwd KpnI-Hlb	ctaGGTACC atgaaacgag gtgtaacaat
Rev EcoRI-Hlb	gcaGAATT C attgtcctattcatttgatg

3.2.3. Polymerase chain reaction (PCR) setup

The following were the final concentration of the master mix used for PCR. The reagent was prepared according to (**Table 3**) below;

Table 3. Preparation of Master mix for PCR.

Reagent	Quantity taken	Final concentration
Taq Buffer	5 µL	1X
$MgCl_2$	4 µL	2Mm
Taq Polimerase	0.3 µL	0.03 U/ Ml
dNTP	1 µL	0.2mM
FWD Primer	0.2 µL	0.4 pmol
RVSE Primer	0.2 µL	0.4 pmol
Deionize water	37.3 µL	-
TOTAL	**50 µL**	

Table 4. PCR setup showing stages of reactions.

First Denaturation	95°C	3 mins	İnitial denaturing of templates
Denaturation	95°C	30 secs	Cycling denaturing step
Annealing	52 °C	30 secs	Primer annealing step
Extension	72°C	1 mins	Product extension step
Cycle	35 times		Number of cycle repetitions
Final Extension	72°C	5 mins	Terminal extension step to ensure all products are maximally extended
Storage step	+4°C		Storage stage

3.2.4. Electrophoresis

A weighted 1.0 g of agarose was dissolved in a 100 mL of 0.5X TBE (Tris-Boric-EDTA) buffer and heated in a microwave (Beko MD 2610). The molten gel was cooled slightly and 5.0 µL of safe view was added and mixed. This is to allow visualization of the DNA in the gel when exposed to U.V. The cooled molten gel was carefully poured into an electrophoresis board (Thermo EC 330), containing toothed comb.

From the PCR amplicon, 5.0 µL was combined with 6X gel dye and loaded onto the agarose gel, along side a λ-*pstI* marker to separate DNA fragments on the basis of size. The results were photographed and analyzed under the transilluminator device (Vilber Lourmat 08 200229). Following the confirmation of successful PCR amplification of gene of interest, cloning was then carried out.

3.3. Cloning of beta hemolysin (*hlb*) gene

The amplicon products and the plasmid (pUC19) were cut by restriction enzymes (*Kpn1* and *EcoRI*) as described in (**Table 5**) below;

Table 5. Restriction reaction mixture showing the quantity of reagent used for both the Amplicon and pUC19 vector.

	Amplicon	**pUC19 vector**
DNA	10 µL	10 µL
Water	6 µL	6 µL
10X Fast Digest Buffer	2 µL	2 µL
KpnI restriction enzyme	1 µL	1 µL
EcoRI restriction enzyme	1 µL	1 µL
Total	**20 µL**	**20 µL**

The following procedures were carried out on ice. The reaction mixtures above were mixed in ependorf tubes and incubated at 37^0C for 25 mins. 5µL from the 20µL were used for electrophoresis to confirm the presence of the DNAs. To attain a volume of 100µL; 85µL of dH_20 was added to the Eppendorf tubes containing the amplicon or vector, separately. 100 µL phenol chloroform (1:1 v/v) was added to both of the tubes containing amplicon and plasmid. The tubes were centrifuged for 5 mins at >10,000 rpm to form a separation of two phases.

Suitable amounts of both prepared vector and insert were combined in 1.5 mL Eppendorf tubes. 10 µL of 3M sodium Acetate (pH 5.2) was added. A volume of 100 µL Isopropanol (1:10 v/v) was added and gently mixed by continually turning the tubes upside down. The samples were left in the freezer for 45 mins to precipitate both the vector and insert DNAs.

The precipitates were centrifuged in a chilled bench top microfuge (MIKRO 200R) at full speed at >13000 rpm for 30 mins at $+4^0$C. The upper phase was decanted leaving the lower phase. A volume of 300µL (%70 ethanol) was added and centrifuge for 5 mins at >13000 rpm. The supernatant was decanted allowing the pellet to dry on a drying sheet at room temperature.

The restricted products were ligated with DNA ligase enzyme and Ligase Buffer. Plasmid-DNA reagent, 10X ligase buffer and Ligase enzyme were collected in an ependorf tubes. The ependorf tubes were incubated with a thermo shaker (MS-100) for 1 hr at 22^0 C, after which it stayed overnight at 16^0 C (Lee et al., 2005).

3.4. Preparation of competent cells

E. coli is the most common bacterial species used in the transformation step of a cloning workflow. The preparation of competent cells in this study was performed using the chemical principle protocol (Sambrook, 2006).

A single fresh colony of DH10B from *E. coli* was taken from an agar plate and inoculated into a liquid medium (5mL) which was incubated at 37^0 C overnight. The next day, 1mL from the 5mL was inoculated into a 50 mL fresh liquid medium (TSB). The inoculated medium was incubated for 3 h to reach the exponential phase of bacterial growth. The medium was incubated in the ice for 30 mins. The falcon tube containing the medium was centrifuged at >5000 rpm $+4^0$ C for 10 mins. The supernatant was decanted, pellet was resuspended with dH_20 and then centrifuge at >5000 rpm at $+4^0$ C for 10 mins again. This process was repeated. 10 mL of cold (%10) glycerol was added to the pellet and centrifuged at >5000 rpm for 10 mins at $+4^0$ C. The supernatant was decanted, re-suspended with 30 mL of cold solution mixture of (80mL $MgCl_2$ and 20mL $CaCl_2$) and centrifuge at >5000 rpm for 10 mins. The supernatant was decanted and 1mL of 0.1M $CaCl_2$ was added and mixed gently to homogenity (Sambrook et al, 2006).

3.5. Transformation

In this study, the chemical transformation technique was used in the cloning experiments. Tubes containing competent cells were thawed at room temperature, dispensed by 100 µL into 1.5 mL Eppendorf tubes and placed in an ice bath. 10 µL of the amplicons were added to each tube, one tube as a control containing 10 µL pUC19 plasmid. The cells were incubated in an ice bath for 30 mins, which was later then heat-pulsed without agitation at 42^oC for 2 minutes and transferred back into the ice bath for 1 minute (Lee et al., 2005).

A volume of 900 µL of sterile Tryptic soy broth (TSB) was added to the tubes which were placed back in the 37^o C incubator and shaken vigorously for 1 hour. A sample of 50 µL and 200 µL of the transformed mixture was poured on the AXI plate (Ampicillin containing medium with X-gal and IPTG). The inoculated petri plates were incubated over-night at 37^0 C. After the growth, at approximately 15 white colonies (i.e. transformants) from the expiremental transformation plates were picked and streaked out on another ampicillin containing LB-agar plate.

The streaked colonies on the second plate were incubated at 37^{o} C overnight and used as templates for colony PCR reaction aimed at identifying transformants harbouring pUC19 plasmids containing the required gene inserts (Sambrook et al., 2006).

3.6. Detection of gene by colony PCR

M13F_ TGT AAA ACG ACG GCC AGT and M**13R_** CAG GAA ACA GCT ATG ACC were used as the forward and reverse primers for the colony PCR. The PCR reaction setup was the same as described above. The amplicons were electrophoresed (30 mins at 100 amp) in 1% agarose gel (TBE 0.5X). The results were photographed and analyzed under the transilluminator device (Vilber Lourmat 08 200229).

Following a successful amplification, the bacterial colony that carried the pUC19 plasmids with required inserts cloned into it could easily be identified after they had been run on a gel. The desired DNA fragments obtained were selected. This permitted one to go back to the second streaked plate and identify the correct clones required to inoculate a liquid culture, thus prepare large quantities for the desired plasmid isolation.

3.6.1. Plasmid DNA isolation

White colonies selected for M13 PCR were inoculated into 5 mL Luria Bertani (LB) broth medium supplemented with Ampicillin (50 mg/mL) and incubated at 37^{0} C for 24 hrs (Pulicherla et al., 2013). After incubation, plasmids from bacterial cultures were isolated by miniprep plasmid isolation kit (Geneaid Presto^{TM} Mini Plasmid Kit) according to the manufacturers protocol:

- From the overnight incubated bacterial culture, 1.5 mL was transfered into microcentrifuge tubes.
- The tubes were centrifuged at 14,000 x g for 1 minute and the supernatant was discarded
- The pelleted bacterial cells were re-suspended in a 200 µL buffer S1 (RNase A added)
- 200 µL of the buffer S3 was added and mixed immediately by inverting the tubes
- Allow the mixture to stand at room temperature for 2 minutes until the lysate is homologous.

- Add 300 µL of the buffer S3 and mix immediately by inverting the tubes 10 times
- Centrifuge at 14,000 x g for 3 minutes. Place a PM column in a collection tube. Add the supernatant to the PM column by decanting or pipetting
- Centrifuge at 14,000 x g for 30 seconds, discard the flow-through and place the PM column back into the same collection tube
- Add 400 µL of the buffer W1 into the PM column tubes and centrifuge at 14,000 x g for 30 seconds
- Discard the flow-through and carefully place the PM column back into the same collection tube
- Add a volume of 600 µL from the buffer W2 into the column and centrifuge for 1 min
- Discard the supernatant and place the PM column back into the same collection tube
- Centrifuge at 14,000 x g again for 2 minutes. Elute the DNA into a sterile epenforf tubes by adding 50 µL of the buffer BE (pH 7.0 and 8.5) to the centre of the PM column
- Allow it to stand for 3 mins and centrifuge for 2 minutes at 14,000 rpm.

Following a successful plasmid purification, *Kpn1* and *EcoR1* restriction enzymes were used for the cutting. For the final confirmation, 5µL of the eluted plasmid was ran on an agarose gel to verify correct size (i.e. insert + vector). The selected clone (i.e. K14) was grown on AXI media which was later stored in 2X skimmed milk at -20^0C.

3.6.2. Biological activity testing of recombinant protein

To observe and test for the biological activity of the recombinant hlb protein, two sheep blood agar plates were used. With a sterilized inoculating loop, Clone K14 containing the recombinant hlb protein was circularly streaked on blood agar plate. A colony from *E. coli* DH10B was used as a control by streaking it perpendicularly to the cloned gene in order to record the haemolytic differences between the two groups. One plate was incubated at 37^0 C for 24 hours. The other plate was incubated at 37^0 C for 18 hours was then transferred to a lower temperature of $+4^0$ C for 6 hours. This phenomenon known as the **''Hot-cold''** reaction was to assess if there will be any significant increasing in haemolytic activity (Smyth et al., 1975). After the subsequent incubation, the plates were examined for the presence of hemolysis.

3.6.3. Sequence Analysis

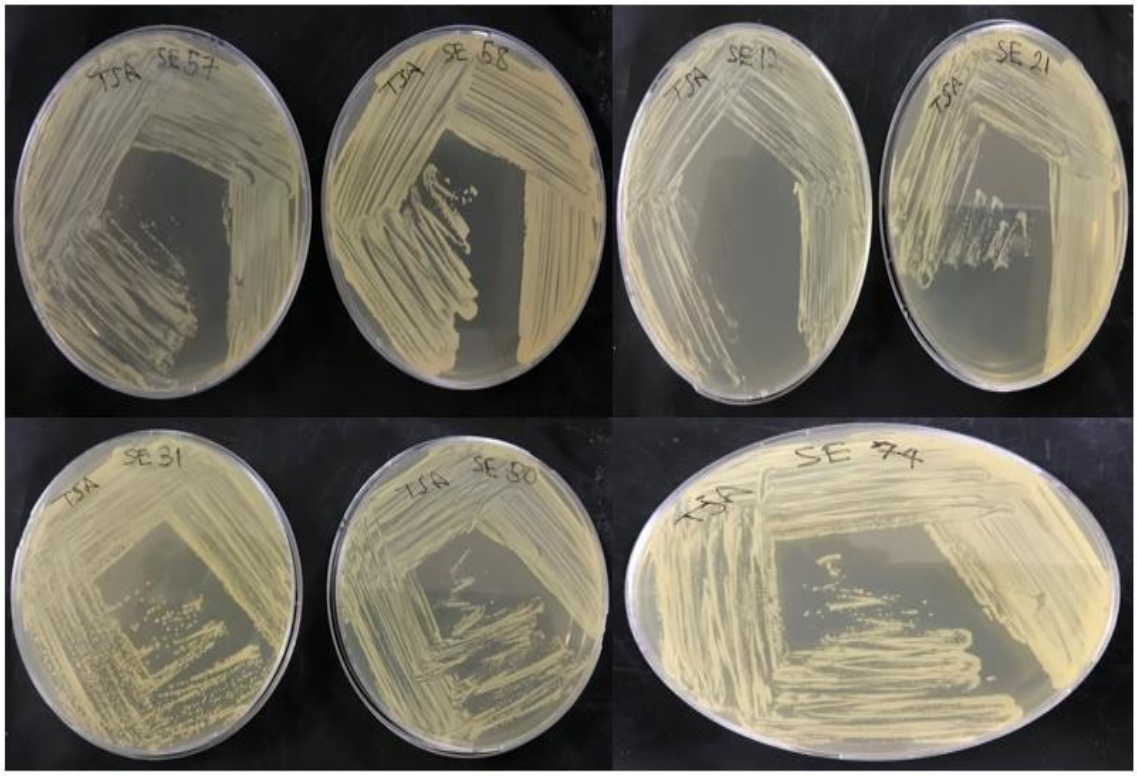
The cloned beta hemolysin gene amplicon of the *Staphylococcus epidermidis* strain was sent for sequencing. The result was then analysed by BLAST DNA at www.ncbi.nlm.nih.gov. From the result of BLAST analysis obtained, similarity rate and the group to which the clone belong was determined.

4. RESULTS

Beta hemolysin gene (*hlb*) represent one of the most studied and important virulence factors in the pathogenicity of Coagulase-negative Staphylococci. The *hlb* gene used in this study was detected among CoNS strains in our laboratory collection, and amplified by using PCR. The amplified hlb gene was then cloned into *E. coli* cells. The hemolytic activity of the recombinant protein was tested on sheep blood agar.

4.1. Propagation of *S. epidermidis* isolates

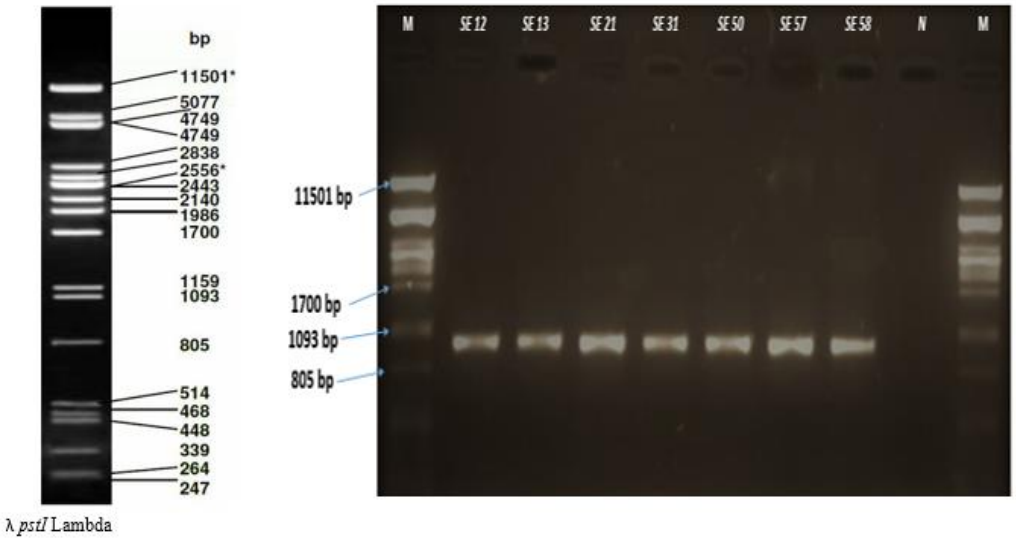
S. epidermidis is a Gram-positive, cocci- shaped and coagulase negative bacterium. The isolates were grown on Tryptic Soy Agar (TSA) plates as shown in (**Picture 1**).



Picture 1. Plates showing various strains of *S. epidermidis* on TSA.

4.2.PCR amplification of beta hemolysin gene

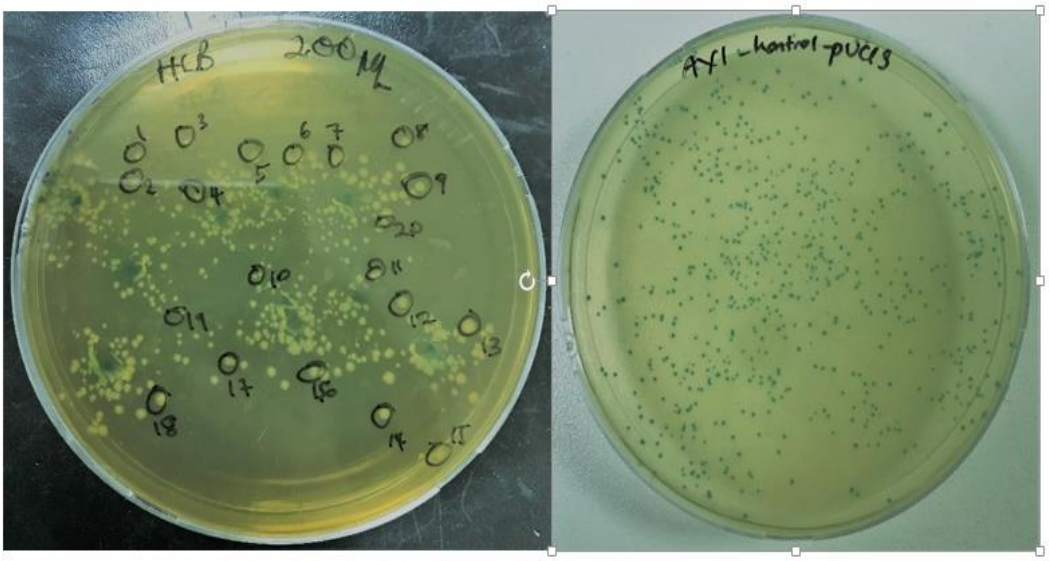
The (**Picture 2**) below, is a (1.0%) agarose gel resolving PCR products generated for cloning. The expected amplicon size was **1005 bp**. From the 7 strains of *S. epidermidis* (*SE12, SE13, SE21, SE31, SE50, SE57* and *SE58*), the PCR result showed positive for all the strains, with N as the negative control. Lambda (λ-pstI) was used as the marker ladder.



Picture 2. Agarose gel showing amplified hlb gene after electrophoresis**.**

4.3.Cloning of beta hemolysin gene

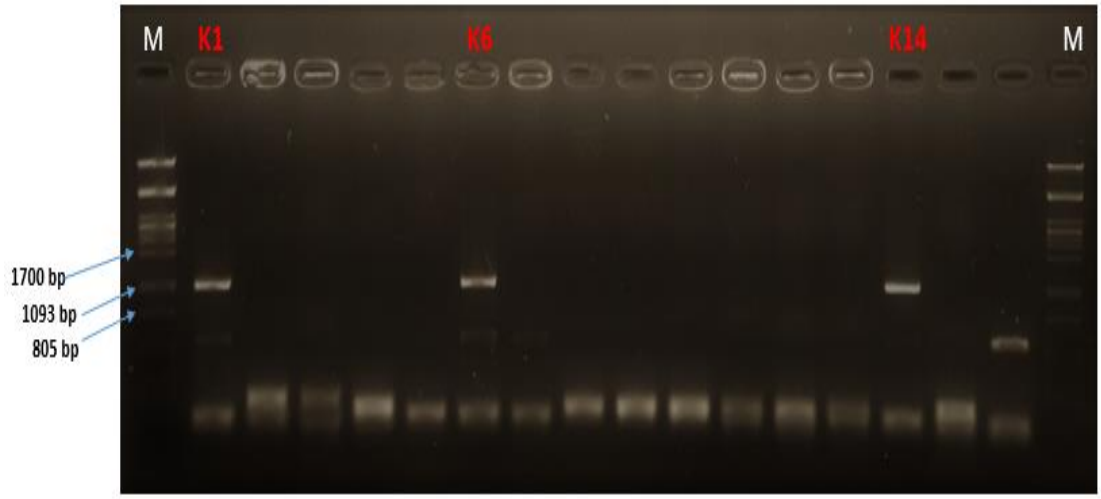
The PCR product of beta hemolysin and the vector (pUC19) were cut with the same restriction enzymes (*KpnI* and *EcoRI*) which was later ligated with T_4 ligase enzyme. After transformation into *E. coli* and selection of successful transformants by growth on ampicillin containing agar plates, a number of colonies were obtained. The white colonies indicate successful gene insertion and blue colonies were the one with self-ligated plasmid.



Picture 3. Blue and White colonies after transformation.
(A) White colonies are the colonies with recombinant plasmid. (B) Control plate with transformed pUC19 plasmid (pUC19) vector only.

4.4. M13 colony PCR

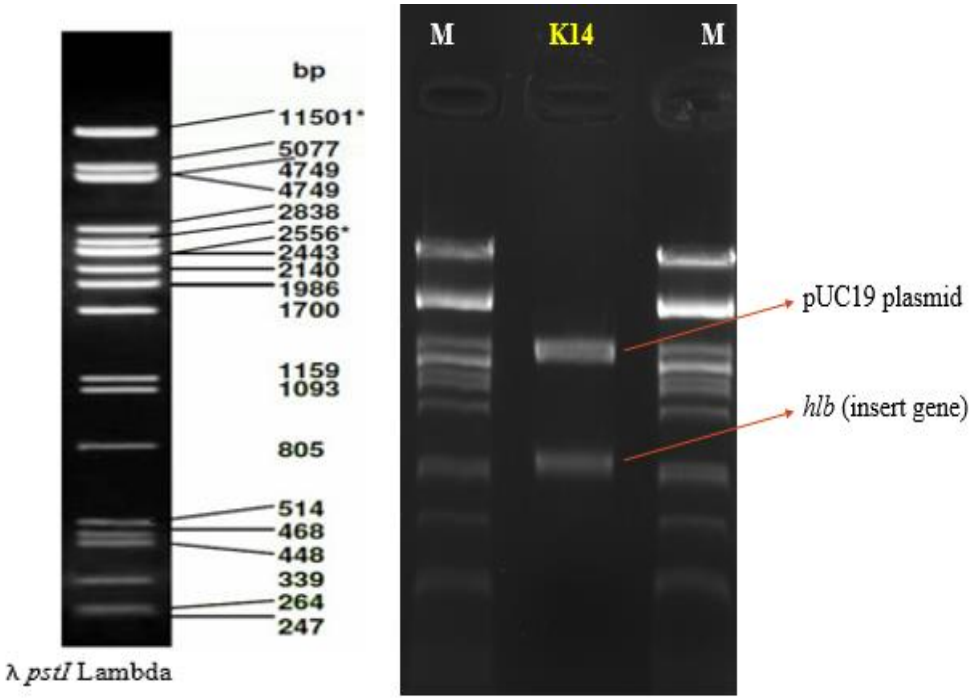
Colony PCR based strategy was performed to determine clones that harboured pUC19 plasmid with the desired gene insert cloned into the Multiple Cloning Site (MCS). From the PCR result obtained, clones (K1, K6 and K14) carried positive band with the exact size (1005 bp) as expected in the (**Picture 4**) below;



Picture 4. PCR-detection for *hlb* positives after M13 PCR.

4.5. Plasmid isolation from recombinant colonies

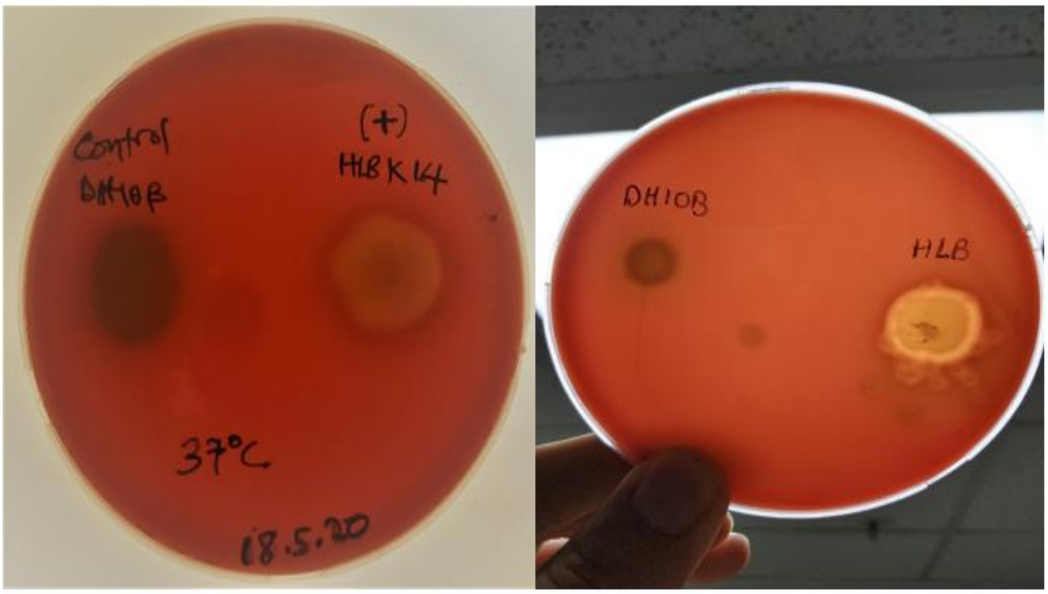
The purified plasmid was cut with *KpnI* and *EcoRI* restriction enzymes and electrophoresed for confirmation. After electrophoresis, two different bands were confirmed, one indicating the insert gene with the other band representing the vector plasmid (pUC19).



Picture 5. PCR- detection of cloned gene after plasmid isolation and restriction.
The top and down arrow denote the vector plasmid (pUC 19) and the insert (*hlb* gene).
M: (λ-*pst*I) marker.

4.6. Observation of hemolysis and hot-cold activity

Recombinant protein obtained in this study was tested on sheep blood agar to assess its haemolytic effect on erythrocytes. The result showed positive for the recombinant protein (i.e. K14), where as no hemolysis observed by DH10B. An increasing hemolysis was observed after the hot-cold phase.



Picture 6. Examining of the effect of recombinat beta hemolysin on sheep blood agar. Hemolysis of erythrocytes was confirmed positve (+) for the clone 14 and negetive (-) for DH10B (control).

4.7. Gene Sequence Analysis

The nucleotide sequence is shown in (**Figure 9**) below. Using BLAST to analyze the nucleotide sequence, our result showed a percentage with a similarity allignment rate of 98.95% (complete genome, *Staphylococcus epidermidis* strain Q47 chromosome). From the chromosome, our gene of interest was ‘‘*sph*’’ within the complement (13198…14202). From this result, it possibly confirms that the gene sequence is the same as the gene of interest.

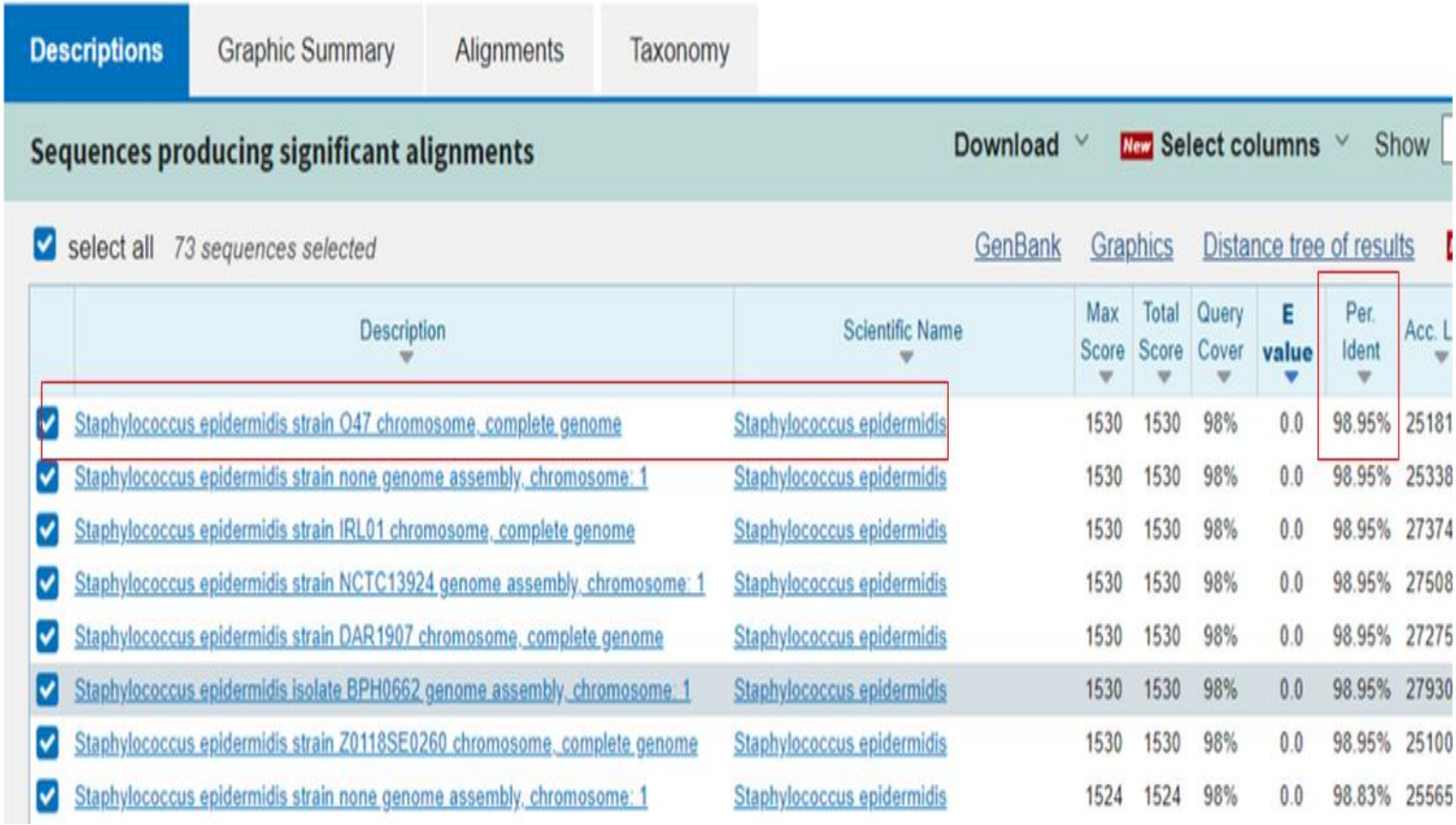


Figure 9. Nucleotide sequence of Beta hemolysin gene by BLAST **(**https://blast.ncbi.nlm.nih.gov/Blast).

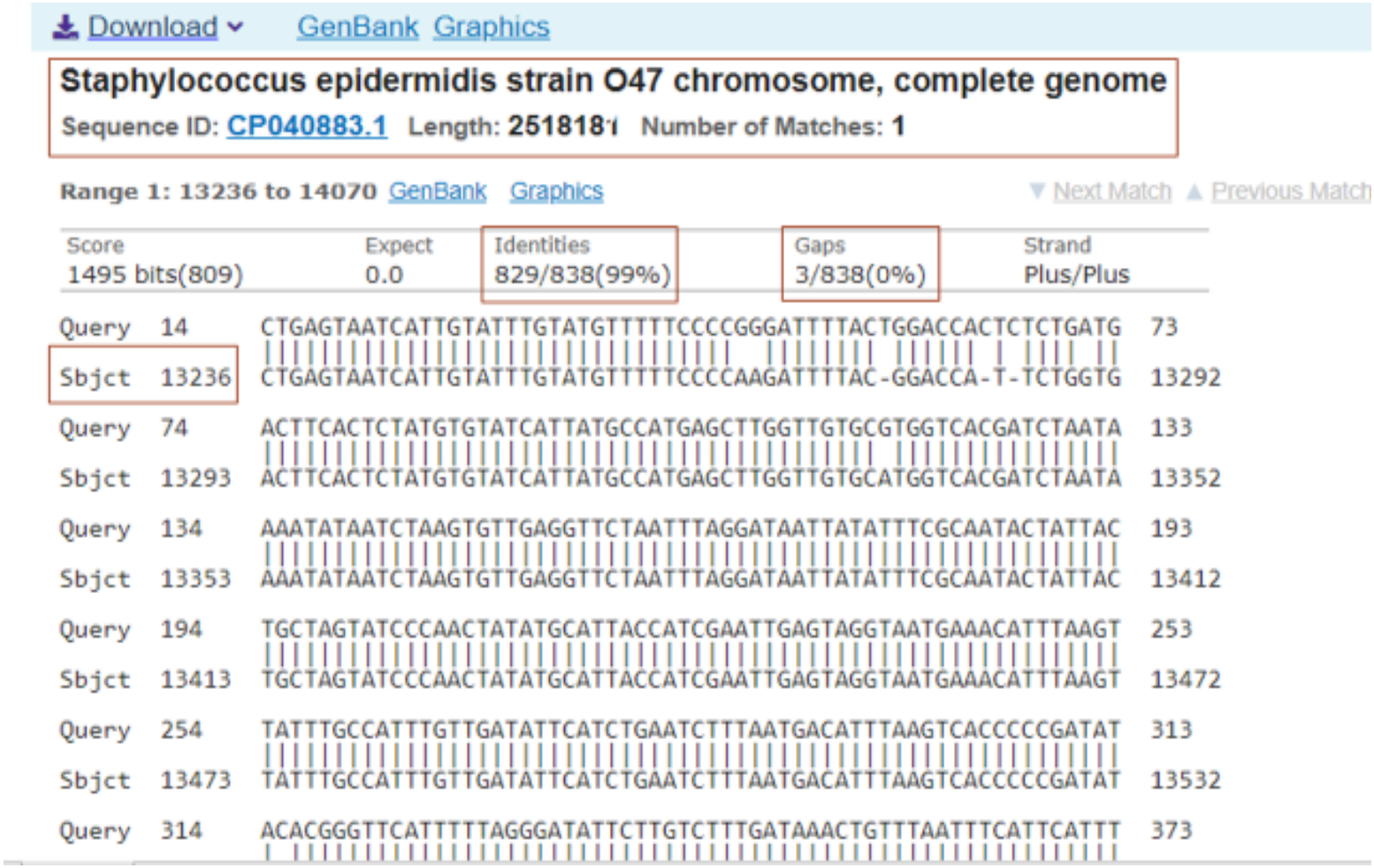


Figure 10. Analysis of Beta hemolysin gene sequence of *Staphylococcus epidermidis* by BLAST **(**https://blast.ncbi.nlm.nih.gov/Blast).

5. DISCUSSION

The continuous increase in human infections by nosocomial pathogens has always been of a great concern among health authorities around the globe. Nosocomial infections occur in patients during health care services or during discharge of patients from hospitals. Extensive studies conducted in the USA and Europe, shows that population at risk are patients allocated in the Intensive Care Unit (ICU) and wound unit, with a population as high as 51% (Hassan et al., 2017).

Coagulase-negative staphylococci represent one of the most frequently identified isolates in the clinical laboratories with it's treatment being very complicated. They are commensal to the various body parts; and can be transferred through contact (Fey, 2010). Based on the examination of the wide variety of CoNS in clinical manifestation, *S. epidermidis* is the most studied species at molecular and genomic level (Otto et al., 2009). The pathogenecity of *S. epidermidis* is very essential due to it's diverse strains and production of lantibiotics; a bacteriocin responsible for bacterial interference on skin and the mucous membrane (Von et al., 2002).

Among the infections contributed by CoNS is the diabetic foot infections (DFI's), which are among the most essential risk factor for lower-extremity amputation, causing substantial morbidity and resulting in high treatment cost (Lipsky et al., 2016). The pathophysiology of DFI's is complicated, related to both host-disorders like, neuropathy and arteriopathy, and also pathogen-related factors like virulence and antibiotic resistance (Dunyach et al., 2016).

Beta hemolysin (*hlb*) is a well characterized exotoxin produced by Staphylococcal strains. The secretion of beta toxin is restricted to a specific strains of bacterium. Recent data give suggestions on the importance of this toxin in inducing apoptosis, cellular disfunction and immunomodulation (Singh et al., 2014).

Our present study was aimed to clone and sequence recombinant *hlb* gene from the isolates of *S. epidermidis* by testing its potential efficacy of hemolysis on sheep blood agar. The *hlb* gene was isolated and detected using PCR with primers specific gene. Positive amplicon was sent for sequencing, after which cloning was carried out using pUC19 plasmid vector. The recombinant protein was tested on sheep blood agar by incubating at 37^0 C, followed by a subsequent temperature at 4^0 C.

Our results successfully exhibited a hemolytic activity on the erythrocytes. The sequence result of the recombinant protein also showed a percentage similarity rate of 98% of (*Staphylococcus epidermidis* strain Q47 chromosome) as expected.

Several screening studies on beta hemolysin (*hlb*) gene has being performed on various dimensions. In our study; the presence of *hlb* gene was positive among the *S. epidermidis* isolates tested. In the study of (Nasaj et al., 2020), *hlb* gene was screen among 91 CoNS isolates from various clinical specimens. Their results showed the presence of *hlb* gene with the highest frequency among *S. epidermidis* (53.80%) and *S. haemolyticus* (39.50%). The results are in accordance to similar results in the studies of (Cunha et al., 2008).

We also observed the hot-cold hemolysis by incubating the plates containing recombinant gene at 37^0 C for 18 hrs, and a subsequent reincubation at 4^o C for 6 hrs. From our result, a hemolytic difference between the plates were observed after the hot-cold phase.

A similar study was performed by (Wioletta et al., 2016), who analyzed the presence of β-toxin by the hot-cold principle from the fifty-one *Staphylococcus pseudintermedius* clinically isolated strains from humans and animals tested.

In a different study, *Okazaki et al.* observed the Hot-cold-hot hemolysis exhibited by Group B *streptococci* (Okazaki et al., 2003). In their study, beta hemolytic zones were exhibited by six human isolates of GBS on agar plates according to different temperatures as shown in (**Figure 11**).

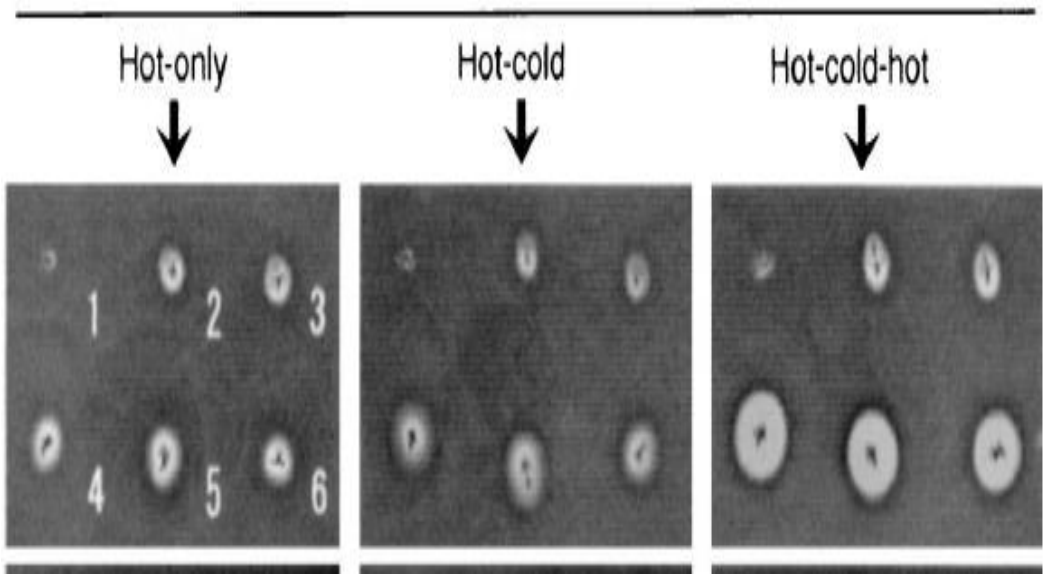


Figure 11. Hemolytic zones exhibited by six GBS on erythrocytes.

At the Hot-only; the plates were incubated at 37^0 C for 24 hrs. For the Hot-cold, the plates were incubated at 37^0 C for 18 hrs, followed by a reincubation at 4^0 C for 6 hrs. Finally at the Hot-cold-hot, the plates were incubated at 37^0 C for 18 hrs, followed by incubation at 4^0 C for 6 hrs and a subsequent reincubation at 37^0 C for 6 hrs. Based on their result, the Hot-cold-hot hemolysis exhibited the GBS was significantly increased on the erythrocytes.

The ‘‘Hot-cold’’ phenomenon is reported in several bacterial species such as *S. aureus* (β-toxin) (Smyth, 1975), *C. perfringens* (alpha-toxin) (Mollby, 1974), *Leptospira interrogans* (Bernheimer, 1986), and *Bacillus cereus* (Ikezawa, 1986).

6. CONCLUSIONS

For the past centuries, bacterial infections continue to impose a serious threat to public health worldwide. Intensified bacterial pathogenesis studies have significantly extended our understanding of the dynamics of disease processes over the last decades at a molecular level. The production and purification of recombinant DNA and recombinant proteins have been of great importance in the the field of medicine and biotechnology. Advanced technology related to recombinant proteins in the diagnosis and treatment of diseases, food industries, agriculture and animal husbandry is being developed day by day.

In this study, the efficacy of our recombinant beta hemolysin (*hlb*) protein produced was tested on sheep blood agar. The procedure used in this study was a simple process, time efficient, low cost, high efficiency, high yield and no side effect. To facilitate future expirements, the recombinant beta toxin will be purified and expressed. The cytotoxicity of this protein will be tested against various cancer cells, like the Hepatocellular carcinoma cells (HepG-2).

This will be a useful key reference in the toxin laboratory and virulence factor gene database. This will provide a unified gateway to store, search, retrieve and update knowledge regarding various virulence factors from different bacterial pathogens.

REFERENCES

A. Salyers, D.D. Whitt Bacterial Pathogenesis: *A Molecular Approach ASM Press, Washington*, 1994.

Arrecubieta, C. et al. SdrF, a *Staphylococcus epidermidis* surface protein, contributes to the initiation of ventricular assist device driveline-related infections. PLoS Pathog e1000411, 5, 2009.

Arrecubieta, C., Lee, M. H., Macey, A., Foster, T. J. & Lowy, F. D. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *J. Biol. Chem*. 2007, 282, 18767–18776.

Baird-Parker AC. The basis for the present classification of staphylococci and micrococci. *Ann. NY Acad. Sci*, 1974, 236, 7-14.

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

Bannerman TL. Staphylococcus, Micrococcs and other catalase-positive coccii that grow aerobically. In: Manual of clinical microbiology, Murray P, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, editors.8th ed. Washington, DC: ASM Press; 2004, p 384 - 404.

Bartlett, A.H.; Hulten, K.G. *Staphylococcus aureus* pathogenesis: Secretion systems, adhesins, and invasins. Pediatr. *Infect. Dis. Journal*. 2010, 29, 860–861.

Bautista, L., Gaya, P., Medina, M. & Nunez, M. A quantitative study of enterotoxin production by sheep milk staphylococci. Appl. Environ. Microb., 1988, 54, 566–569.

Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. *Clin Microbiol Rev*. 2014, 27(4):870-926.

Becker, K.; Heilmann, C.; Peters, G. Coagulase-negative staphylococci. *Clin. Microbiology.* Rev., 2014, 27, 870–926.

Belkaid, Y., and Tamoutounour. The influence of skin microorganisms on cutaneous immunity. Nat. Rev. Immunol., 2016, 16, 353–366. doi: 10.1038/nri.2016.48.

Bernheimer, A. W., and R. F. Bey. Copurification of Leptospira interrogans serovar pomona hemolysin and sphingomyelinase C. Infect. Immun., 1986, 54**,** 262–264.

Bien J, Sokolova O, Bozko P. Characterization of virulence factors of Staphylococcus aureus: novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response. J Pathogens, 2011. doi.org/10.4061/2011/601905.

Boyce JM. Epidemiology and prevention of nosocomial infections. In: The Staphylococci in Human Disease, KB Crossley, GL Archer, New York: Churchill Livingston, 1997,ed. 1:309.29.

Brennan, M. P., Loughman, A., Devocelle, M., Arasu, S., Chubb, A. J., Foster, T. J., et al. Elucidating the role of *Staphylococcus epidermidis* serine-aspartate, 2009.

Centres for Disease Control and Prevention, U.S. Department of Health and Human Services, Antibiotic resistance threats in the United States, Atlanta, GA 2013, http://www.cdc.gov/ drugresistance/pdf/ar-threats-2013-508.pdf, 2019.

Cheung GY, Duong AC and Otto M. Direct and synergistic hemolysis caused by Staphylococcus phenol-soluble modulins: implications for diagnosis and pathogenesis. Microbes Infect., 2012, 14:380-6.

Cosgrove, S.E., and Carmeli, Y. The impact of antimicrobial resistance on health and economic outcomes. Clin Infect Diseases, 2003, 36, 1433-1437.

Costerton, J.W., et al. Bacterial biofilms: a common cause of persistent infections. Science 1999, 284, 1318-1322.

Cross A. S. What is a virulence factor? Critical care (London, England), 2008, 12 (6), 196. https://doi.org/10.1186/cc7127.

Cunha, Calsolari, R. A. O. Toxigenicity in *Staphylococcus aureus* and Coagulase-Negative Staphylococci: Epidemiological and Molecular Aspects. Microbiology Insights, 2008, MBI.S796. doi:10.4137/mbi.s796

Diekema, D. J., Pfaller, M. A., Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N., et al. Clin. Infect. Dis., 2001, 32(Suppl. 2), 114–132. doi: 10.1086/320184.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. Exotoxins of *Staphylococcus aureus*. Clinical Microbiology Reviews, 2000, 13, 16-34. https://doi.org/10.1128/CMR.13.1.16.

Doery HM, Magnuson BJ, Galasekharam J, Pearson JE. The properties of phospholipase enzymes in staphylococcal toxins. *J. Gen Microbiol.,* 1965, 40, 283–96.

Dunyach-Remy C, Ngba Essebe C, Sotto A, et al. *Staphylococcus aureus* toxins and diabetic foot ulcers: role in pathogenesis and interest in diagnosis. Toxins (Basel), 2016; doi:10.3390/toxins8070209.

ECDC. European Centre for Disease Prevention and Control: Annual Epidemiological Report 2013: Reporting on 2011 surveillance data and 2012 epidemic intelligence data, 2013.

Fairbrother RW. Coagulase production as a criterion for the classification of the staphylococci. J Pathol Bacteriol. 1940, 50(1), 83-88.

Faurschou, M. & Borregaard, N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect., 2003, 5, 1317–1327.

FD. Staphylococcus aureus infections. N. Engl. J. Med., 1998, 339(8): 520-32.

Fey PF, Olson ME. Current concepts in biofilm formation of *Staphylococcus epidermidis.* Future Microbiol., 2010. doi.org/10.2217/fmb.10.56.

Flemming, H.C., and Wingender, J. The biofilm matrix. Nat Rev Microbiol, 2010, 8, 623-633.

Flores-Díaz, M.; Monturiol-Gross, L.; Naylor, C.; Alape-Girón, A.; Flieger, A. Bacterial Sphingomyelinases and Phospholipases as Virulence Factors. Microbiol. Mol. Biol. Rev. 2016, 80, 597–628.

Freer, J.H. and Arbuthnott, J.P.. Toxins of *Staphylococcus aureus*. Pharmacol. Ther., 1983, 19, 55–106.

Futerman, A.H. and Hannun, Y.A. The Complex Life of Simple Sphingolipids. EMBO Reports, 2004, 5, 777-782. doi.org/10.1038/sj.embor.7400208.

Glenny, A.T., Stevens, M.F. Staphylococcus toxins and antitoxins. J. Pathol., 1935, 40, 201–210. Toxins 2018, 10, 252 15 of 19.

Goni, F.M. and Alonso, A. Sphingomyelinases: Enzymology and Membrane Activity. FEBS Letters, 2002, 531, 38-46. doi.org/10.1016/S0014-5793(02)03482-8.

Gouaux, J.E.; Braha, O.; Hobaugh, M.R.; Song, L.; Cheley, S.; Shustak, C.; Bayley, H. Subunit stoichiometry of staphylococcal alpha-hemolysin in crystals and on membranes: A heptameric transmembrane pore. Proc. Natl. Acad. Sci. USA, 1994, 91, 12828–12831.

Gravet A, Colin DA, Keller D, et al. Characterization of a novel structural member, LukELukD, of the bi-component staphylococcal leucotoxins family.FEBS Lett, 1998.

Gray, G.S. and Kehoe, M. Primary sequence of the α-toxin gene from S*taphylococcus aureus*. Wood 46. Infect. Immun., 1984, 46, 615–618.

Grumann, D.; Nübel, U.; Bröker, B.M. *Staphylococcus aureus* toxins—Their functions and genetics. Infect. Genet. Evol., 2014, 21, 583–592.

Harder, J. & Schroder, J. M. Antimicrobial peptides in human skin. Chem. Immunol. Allergy 2005, 86, 22–41.

Harshman, S., Boquet, P., Dufl ot, E., Alouf, J.E., Montecucco, C. and Papini, E.. Staphylococcal -toxin: a study of membrane penetration and pore formation. J. Biol. Chem., 1989, 264:14978–84.

Hassan Ahmed Khan, Fatima Kanwal Baig, Riffat Mehboob, Nosocomial infections: Epidemiology, prevention, control and surveillance, *Asian Pacific Journal of Tropical Biomedicine*, Volume 7, Issue 5, 2017, https://doi.org/10.1016/j.apjtb.2017.01.019.

Hébert GA, Hancock GA. Synergistic hemolysis exhibited by species of staphylococci. *J Clin Microbiol.*, 1985.

Hedstrom, S.A. and Malmqvist, T. Sphingomyelinase activity of *Staphylococcus aureus* Strains from Recurrent Furunculosis and Other Infections. Acta Pathologica Microbiologica Scandinavica Series B : Microbiology, 90B, 1982, 217-220, https://doi.org/10.1111/j.1699 0463.1982.tb00108.

Heilmann, C. et al. Identification and characterization of a novel autolysin (Aae) with adhesive properties from Staphylococcus epidermidis. Microbiology, 149, 2003, 2769–2778.

Heilmann, C., Hussain, M., Peters, G. & Gotz, F. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Mol. Microbiol., 1997, 24, 1013–1024.

Herman, A. Superantigens: Mechanism of T-cell stimulation and role in immune responses. Annu. Rev. Immunol., 1991, 9:745–72.

Honeyman, A.; Friedman, H.; Bendinelli, M. Staphylococcus aureus Infection and Disease; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2006.

Huseby, M., Shi, K., Brown, C.K., Digre, J., Mengistu, F., Seo, K.S., et al. Structure and Biological Activities of β Toxin from *Staphylococcus aureus*. Journal of Bacteriology, 2007, 189, 8719-8726, https://doi.org/10.1128/JB.00741-07.

Ikezawa, H., M. Matsuhita, M. Tomita, and R. Taguchi. Effect of metal ions on sphingomyelinase activity of *Bacillus cereus*. Arch. Biochem. Biophys, 1986, 249**:**588–595.

Izano, E. A., Amarante, M. A., Kher, W. B., and Kaplan, J. B. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and *Staphylococcus epidermidis* biofilms. Appl. Environ. Microbiol., 2008, 74, 470–476. doi:10.1128/AEM.02073-07.

Joseph Sambrook and David W. Russell. Cold Spring Harb Protoc, 2006, dio: 10.1101/pdb.prot3932.

K. Bush and G. A. Jacoby, "Updated functional classification of β-lactamases," Antimicrobial Agents and Chemotherapy, 2010, 54, 969–976.

Kloos WE. Taxonomy and systematics of staphylococci indigenous to humans. In: The Staphylococci in Human Disease, KB Crossley, GL Archer, New York: Churchill Livingston 1997, ed 1:113-37.

Kloos, W. E., and Bannerman, T. L.. Staphylococcus and Micrococcus. In Manual of Cinical Microbiology, 1995, 6th edition, Pp. 282- 298.

Koneman, E.W. Color Atlas and Textbook of Diagnostic Microbiology. Philadelphia: J.B. Lippincott, 1997, 5th edition.

Kusch H, Engelmann S. Secrets of the secretome in Staphylococcus aureus. Int J Med Microbiol., 2014, 304, 133–141.

L. B. Rice, "Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE," *Journal of Infectious Diseases*, 2008, vol. 197, no. 8, pp. 1079–1081.

L.B.Rice,"Progress and challenges in implementing the research on ESKAPE pathogens," Infection Control and Hospital Epidemiology, 2010.

Lai, Y., Di Nardo, A., Nakatsuji, T., Leichtle, A., Yang, Y., Cogen, A. L., et al. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nat. Med. 2009, 15, 1377–1382. doi: 10.1038/nm.2062.

Larsen, H.D., Aarestrup, F.M. Jensen. N.E. Geographical Variation in the Presence of Genes Encoding Superantigenic Exotoxins and β-Hemolysin among *Staphylococcus aureus* isolated from Bovine Mastitis in Europe and USA. Veterinary Microbiology, 2002, 85, 61-67. https://doi.org/10.1016/S0378-1135(01)00478-3.

Lee JS, Hadjipanayis AG, Parker MD. Viral vectors for use in the development of biodefense vaccines. Adv Drug Deliv Rev.; 2005, 57, 1293-314.

Lina, G. et al. Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles. Appl. Environ. Microbiol., 2003, 69, 18–23.

Lipsky BA, Aragon-Sanchez J, Diggle M, et al. IWGDF guidance on the diagnosis and management of foot infections in persons with diabetes. Diabetes Metab Res Rev. 2016, 32(Suppl 1):45–74.

Löfer, B. et al. *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. PLoS Pathog., 2010, 6, e1000715.

Lukas Stulik , Stefan Malafa , Jana Hudcova , Harald Rouha , Bence Z. Henics , Donald E. Craven , Agnes M. Sonnevend , and Eszter Nagy, α-Hemolysin Activity of Methicillin-Susceptible *Staphylococcus* aureus Predicts Ventilator-associated Pneumonia, 2014.

M.Singh, A. Singh A.Sharma. Production of an N-terminal –truncated recombinant beta-hemolysin from *Staphylococcus aureus*, 2014, 42; 191-198.

Ma, X. X. et al. Novel type of staphylococcal cassette chromosome mec identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob. Agents Chemother, 2002, 46, 1147–1152.

Mah, T.F., and O'Toole, G.A. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol., 2001, 9, 34-39.

Marin, M. E., de la Rosa, M. C. & Cornejo, I. Enterotoxigenicity of Staphylococcus strains isolated from Spanish dry-cured hams. Appl. Environ. Microbiol, 1992, 58, 1067–1069.

McCrea, K. W. et al. The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology, 2000, 146, 1535–1546.

Medora Huseby, Ke Shi, C. Kent Brown, Jeff Digre, Fikre Mengistu, Keun Seok Seo, Gregory A. Bohach, Patrick M. Schlievert, Douglas H. Ohlendorf and Cathleen A. Earhart. Structure and Biological Activities of Beta Toxin from *Staphylococcus aureus*, *Journal of bacteriology*, 2007, 189: 8719–8726. doi:10.1128/JB.00741.

Meduski, J. W., and P. Hochstein. Hot-cold hemolysis: the role of positively charged membrane phospholipids, 1972, 15, 565-566.

Mehlin, C.; Headley, C.M.; Klebanoff, S.J. An inflammatory polypeptide complex from *Staphylococcus epidermidis*: Isolation and characterization. J. Exp. Med, 1999, 189, 907–918,

Miragaia, M., Couto, I. & de Lencastre, H. Genetic diversity among methicillin-resistant *Staphylococcus epidermidis* (MRSE). Microb. Drug Resis, 2005, 11, 83–93.

Mollby, R., T. Wadstrom, C. J. Smyth, and M. Thelestam.. The interaction of phospholipase C from *Staphylococcus aureus* and *Clostridium perfringens* with cell membranes. *J. Hyg. Epidemiol. Microbiol. Immunol.*, 1974, 18: 259–270.

Nasaj, M., Saeidi, Z., Asghari, B., Roshanaei, G., & Arabestani, M. R. Identification of hemolysin encoding genes and their association with antimicrobial resistance pattern among clinical isolates of coagulase-negative Staphylococci, 2020, doi:10.1186/s13104-020-4938-0

Nilsson, M. et al. A fibrinogen-binding protein of *Staphylococcus epidermidis*. Infect. Immun. 1998, 66, 2666–2673.

Noble WC, Pitcher DG.. Microbial ecology of the human skin. Adv. Microbiol. Ecol. 1978, 2, 245-53

O'Callaghan, R.J., Callegan, M.C., Moreau, J.M., Green, L.C., Foster, T.J., Hartford,O.M., et al. Specific Roles of β-Toxin and β-Toxin during *Staphylococcus aureus* Corneal Infection. Infection and Immunity 1997, 65, 1571-1578.

O'Grady, N. P. et al. Guidelines for the prevention of intravascular catheter-related infections. MMWR Recomm. Rep, 2002, 51, 1–26.

Okazaki, N., Osawa, R., Suzuki, R., Nikkawa, T., & Whiley, R. A. Novel Observation of Hot-Cold-Hot Hemolysis Exhibited by Group B Streptococci. *Journal of Clinical Microbiology*, 2003, 41(2), 877–879. doi:10.1128/jcm.41.2.877-879.

O'Neill, Antimicrobial resistance: Tackling a crisis for the health and wealth of nations, 2019, http://amr-review.org/Publications.

O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm formation as microbial development. Annu. Rev. Microbiol., 2000, 54, 49–79.

Otto M. Staphylococcus colonization of the skin and antimicrobial peptides. Expert Rev Dermatology, 2010; 5(2):183–95. https://doi.org/10.1586/edm.10.6.

Otto, M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu. Rev. Microbiol., 2010, 64, 143–162.

Otto, M. *Staphylococcus epidermidis*—The "accidental" pathogen. Nat. Rev. Microbiol. 2009; 7, 555–567.

Park, P. W., Rosenbloom, J., Abrams, W. R., Rosenbloom, J. & Mecham, R. P. Molecular cloning and expression of the gene for elastin-binding protein (ebpS) in *Staphylococcus aureus. J. Biol. Chem*. 271, 1996, 15803–15809.

Perret M, Badiou C, Lina G, Burbaud S, Benito Y, Bes M, et al. Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner. Cell Microbiol., 2012.

Pinheiro, L., Brito, C. I., Pereira, V. C., Oliveira, A., Bartolomeu, A. R., Camargo, C. H., et al. , 2016, doi: 10.1089/mdr.2015.0064.

Pittet, D., et al. Infection control as a major World Health Organization priority for developing countries. *J Hosp Infect.,* 2008, 68, 285-292.

Projan, S.J.; Kornblum, J.; Kreiswirth, B.; Moghazeh, S.L.; Eisner, W.; Novick, R.P. Nucleotide sequence. The beta-hemolysin gene of *Staphylococcus aureus*. Nucleic Acids Res. 1989, 17, 3305.

Rasigade JP, **Vandenesch F.** *Staphylococcus aureus*: a pathogen with still unresolved issues. Infect. Genet. Evol., 2014.

Raue, S., Fan, S.-H., Rosenstein, R., Zabel, S., Luqman, A., Nieselt, K., & Götz, F. The Genome of *Staphylococcus epidermidis* O47. Frontiers in Microbiology, (2020). 11. doi:10.3389/fmicb.2020.02061.

Rock M, Buntain BJ, Hatfield JM, et al. Animal-human connections, "one health," and the syndemic approach to prevention. Soc Sci Med.2009;68(6):991-5. DOI:10.1080/14787210.2020.1730813.

Rogers, K. L., Fey, P. D., and Rupp, M. E. Coagulase-negative staphylococcal infections. Infect. Dis. Clin. North Am., 2009, 23, 73–98. doi: 10.1016/j.idc.2008.10.001.

Rosenbach FJ. Micro-Organismen bei den Wund-Infections-Krankheiten des Menschen. Wiesbaden: J. F. Bergmann, 1884.

S. S. Magill, J. R. Edwards, W. Bamberg et al,. "Multistate point-prevalence survey of health care-associated infections," The New England Journal of Medicine, 2014, 370, no. 13, pp. 1198–1208.

Salgado-Pabón W, Herrera A, Vu BG, Stach CS, Merriman JA, Spaulding AR, Schlievert PM. Staphylococcus aureus b-toxin production is common in strains with the b-toxin gene

inactivated by bacteriophage. *J Infect Dis* , 2014, 210, 784–792,

Scharschmidt, T. C., Vasquez, K. S., Truong, H. A., Gearty, S. V., Pauli, M. L., Nosbaum, A., et al. A wave of regulatory T cells into neonatal skin mediates tolerance to commensal microbes. Immunity, 2015, 43, 1011–1021. doi: 10.1016/j.immuni.2015.10.016.

Sireesha Divyakolu, Rosy Chikkala, Kamaraju Suguna Ratnakar, Venkataraman Sritharan, Hemolysins of *Staphylococcus aureus*—An Update on Their Biology, Role in Pathogenesis and as Targets for Anti-Virulence Therapy, 2019.

Smith, M.P. Staphylococcus γ-Hemolysin. *The Journal of Pathology*, 1938, 47, 379-393. https://doi.org/10.1002/path.1700470303.

Smyth, C. J., R. Mollby, and T. Wadstrom. Phenomenon of hot-cold hemolysis: chelater-induced lysis of sphingomyelinase-treated erythrocytes. Infect. Immun. 12**:** 1975, 1104–1111.

Song, L.; Hobaugh, M.R.; Shustak, C.; Cheley, S.; Bayley, H.; Gouaux, J.E. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science, 1996, 274, 1859–1866.

Spaan, A. N., van Strijp, J. A. G. & Torres, V. J. Leukocidins: staphylococcal bi-component pore-forming toxins fnd their receptors. Nat. Rev. Microbiol., 2017, 15, 435–447, https://doi.org/10.1038/nrmicro.2017.27.

Sutherland, I.W. The biofilm matrix - an immobilized but dynamic microbial environment. Trends Microbiol, 2001, 9, 222-227.

Tam, K., & Torres, V. J. *Staphylococcus aureus* Secreted Toxins and Extracellular Enzymes. Gram-Positive Pathogens, 2019, 640–668. doi:10.1128/9781683670131.ch40.

Tenover, F.C. Mechanisms of antimicrobial resistance in bacteria. *Am J Infect Control*, 2006, 34, S3-10; discussion S64-73.

Trampuz, A., and Zimmerli, W. Prosthetic joint infections: update in diagnosis and treatment.

Swiss Med. Wkly, 2005, 135, 243–251. doi: 10.4414/smw.2005.10934.

Uckay, I. et al. Foreign body infections due to *Staphylococcus epidermidis*. Ann. Med, 2009, 41, 109–119.

Ventura CL, Malachowa N, Hammer CH, et al. Identification of a novel *Staphylococcus aureus* two-compèonent leukotoxin using cell surface proteomics. PLoS ONE 2010, 5:e11634.https://doi.org/10.1371/journal.pone.0011634.

Von Eiff, C., Peters, G., & Heilmann, C. Pathogenesis of infections due to coagulase negative staphylococci. The Lancet Infectious Diseases, 2002. doi:10.1016/s1473-3099(02)00438-3.

Vuong, C., Kocianova, S., Yu, J., Kadurugamuwa, J. L., and Otto, M. Development of real-time in vivo imaging of device-related *Staphylococcus epidermidis* infection in mice and influence of animal immune status on susceptibility to infection. *J. Infect. Dis*., 2008, 198, 258–261. doi: 10.1086/589307.

Wang JH, Niu HY, Zhang M, He P, Zhang Y and Kan L. Apoptosis induced by *Staphylococcus aureus* in human monocytic U937 cells involves Akt and mitogen-activated protein (MAPK) phosphorylation. *African Journal of Biotechnology*, 2011, 10(21): 4318-4327.

Wang X, Noda M, Kato I. Stimulatory effect of staphylococcal leukocidin on phosphoinositide metabolism in rabbit polymorphonuclear leukocytes. Infect Immun., 1990, 58, 2745–9.

Wang, R.; Braughton, K.R.; Kretschmer, D.; Bach, T.-H.L.; Queck, S.Y.; Li, M.; Kennedy, A.D.; Dorward, D.W.; Klebanoff, S.J.; Peschel, A.; et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med., 2007, 13, 1510–1514.

Weinstein, R.A., and Darouiche, R.O. Device-associated Infections: a macroproblem that starts with microadherence, *Clin Infect Dis.*, 2001, 33, 1567-1572.

Welch WH. Conditions underlying the infection of wounds. *Am J Med Sci.* 1891;102(5):439-

465.

Wenzel, R.P., and Edmond, M.B. The impact of hospital-acquired bloodstream infections. Emerg Infect Dis., 2001, 7, 174-177.

Wilkinson BJ. Biology. In The Staphylococci in Human Disease, ed. KB Crossley, GL Archer, New York: Churchill Livingston, 1997, 1:1.38.

Wioletta Kmieciak, Eligia M.Szewczyk, Marcin Ciszewski. Searching for β-Haemolysin hlb gene in *S. pseudintermedius* with species-specific primers, 2016, 73, 148-152.

Wiseman, G. M. In: Ajl, S. J. et al. (ed.), Microbial toxins, a comprehensive treatise, Academic Press Inc., New York, 1970, vol. 3, chapt. 6.

Yamashita, K.; Kawai, Y.; Tanaka, Y.; Hirano, N.; Kaneko, J.; Tomita, N.; Ohta, M.; Kamio, Y.; Yao, M.; Tanaka, I. Crystal structure of the octameric pore of staphylococcal -Hemolysin reveals the _-Barrel pore formation mechanism by two components. Proc. Natl. Acad. Sci. USA, 2011, 108, 17314–17319.

Yao, Y., Sturdevant D. E., and Otto, M. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol soluble modulins in formation of biofilms. *J. Infect. Dis.*, 2005, 191, 289–298. doi: 10.1086/426945.

Yoong, P.; Torres, V.J. The effects of *Staphylococcus aureus* leukotoxins on the host: Cell lysis and beyond. Curr. Opin. Microbiol., 2013, 16, 63–69.

Zhang, Y.-Q., Ren, S.-X., Li, H.-L., Wang, Y.-X., Fu, G., Yang, J.,Wen, Y.-M. Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). Molecular Microbiology, 2003, 49(6), 1577–1593. doi:10.1046/j.1365.

CURRICULUM VITAE

Personal Information

Surname, Name	: ALI, Sahd
Nationality	: Ghanaian
Place and Date of Birth	: Accra / 08.07.1991
E-mail	: sahdali24@gmail.com _ashescaptain@gmail.com _1824800105@stu.adu.edu.tr
Foreign Languages	: English , Turkish

EDUCATİON

Degree	Institution	Graduation Date
Masters	Aydin Adnan Menderes University	2020
Undergraduate	Aydin Adnan Menderes University	2018
High school	Ejuraman Anglican Senior High School	2012

SCHOLARSHIPS AND AWARDS

xxx

WORK EXPERIENCE

YEAR	ORGANISATION	TITLE
2016-2017	Sea Turtle Conservation, Patara, ANTALYA	Intern

ACADEMIC PUBLICATIONS

1. ARTICLES

xxx

2. PROJECTS

Cloning of Beta Hemolysin Gene From Coagulase Negative Staphylococci and Testing of its Biological Activity. BAP Project: FEF-19021.

3. CONGRESS PAPERS

A) Papers Presented at International Congresses

Amplification and Cloning of Hemolytic phospholipase C (*plcH*) gene from *Pseudomonas aeruginosa*.

B) Papers Presented at National Congresses

xxx