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

SAHD ALI
MASTER'S PROGRAM THESIS

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LIST OF ABBREVIATIONS

AXI	Ampisilin, X-gal, IPTG
β	Beta
hly	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 <i>mec</i>
SE	<i>Staphylococcus epidermidis</i>
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
X-Gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

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ÖZET

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

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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 (*hly*), *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 (*hly*) proteinin aktivitesini ele almak için diyabetik ayak enfeksiyonlarından (DAI) alınan örneklerden *S. epidermidis* suşları kullanılmıştır. *hly* 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 (*hly*), Koagülaz negatif stafilokoklar (KNS), rekombinant gen, sfingomyelinaz, sitotoksin.

ABSTRACT

CLONING OF BETA HEMOLYSIN GENE FROM COAGULASE NEGATIVE 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 (*hly*) 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 *hly* 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 (*hly*), 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 industrialized 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 antimicrobial drugs. This resistance creates a limitation on antibiotic therapy for the hard-to-treat multi-drug resistant bacteria. 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 (Salysers 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 (*hlyB*) 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 (*hlyB*) 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 *hlyB* protein.

2. LITERATURE REVIEW

2.1. Classification of Coagulase-negative staphylococci (CoNS)

Since the early beginning 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).

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

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

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

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

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

S. pseudoxylus

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 pathogenicity is distinguished by the production of its 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).

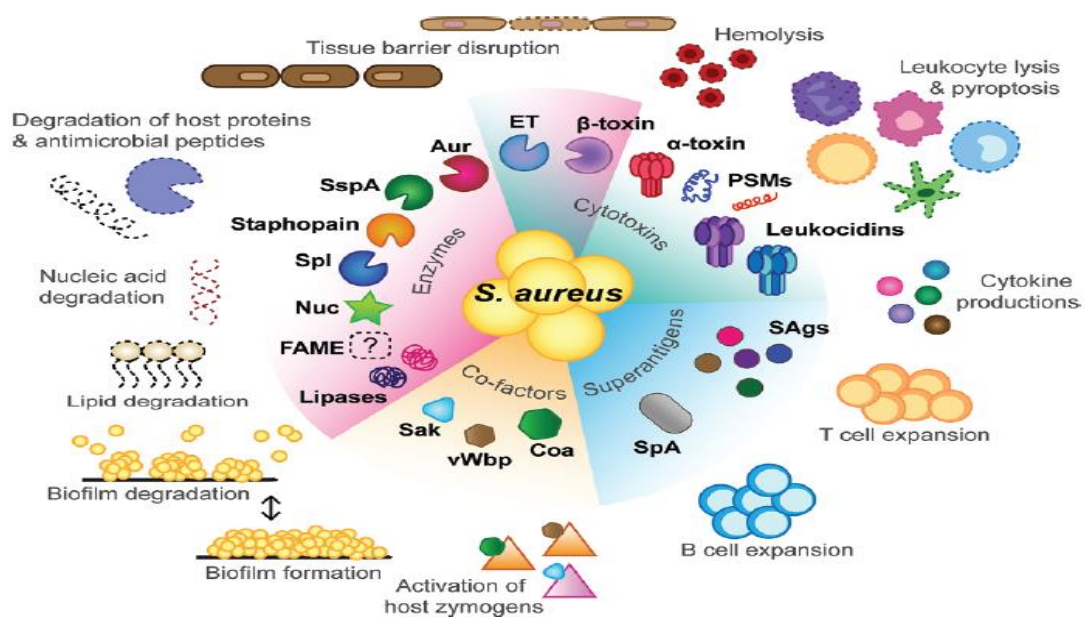


Figure 1. Virulence factors secreted by *S. aureus* (Tam and Torres, 2019).

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 separate 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 inflammasome, a cytolytic multiprotein oligomer (Perret et al., 2012).

2.2.3. Pantone-valentine leukocidin

Pantone-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 pantone-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, neutrophils), 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-threatening (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).

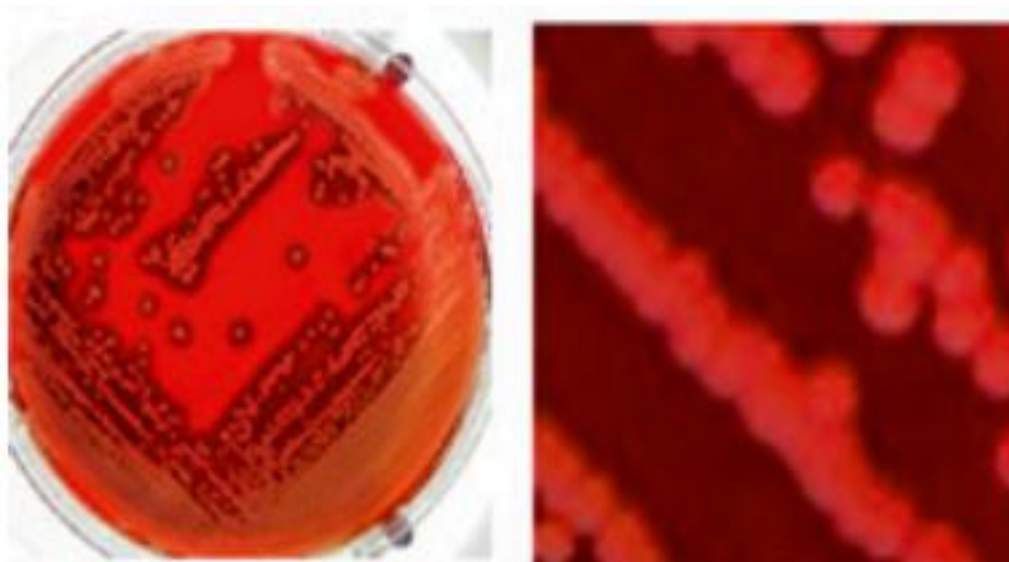


Figure 2. Activity of Alpha toxin on sheep blood agar (Lukas et al., 2014).

2.3.2. Beta hemolysin (β - toxin)

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

Beta hemolysin is a Mg^{2+} dependent sphingomyelinase C. Sphingomyelinases 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 pathogenicity of staphylococcal bacteria was not known in the previous years. Recent studies however, have now postulated the understanding of beta hemolysin, due to its effective activity on animal isolates (Dinges et al., 2000).

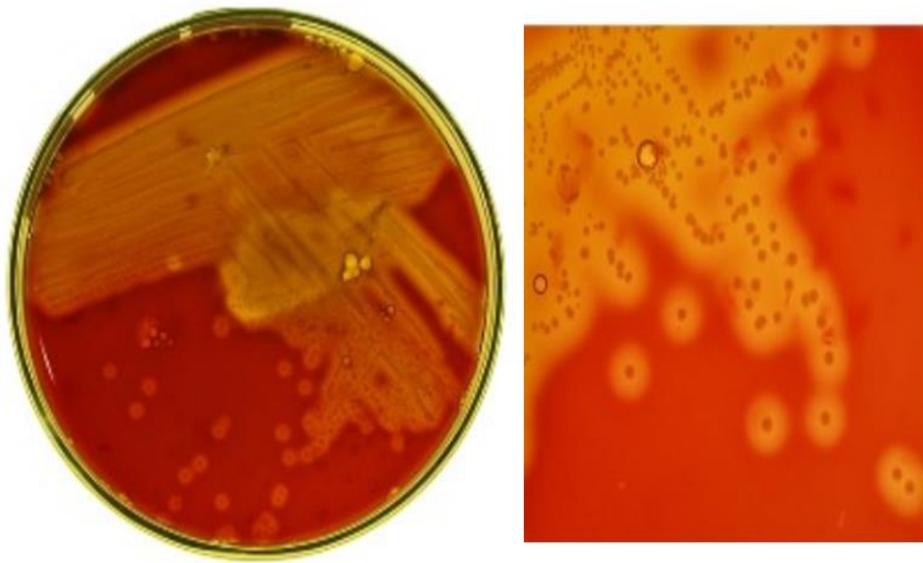


Figure 3. Beta toxin activity on blood agar (Niederstebruch et al., 2017).

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°C after treatment at 37°C. Much has been written about the mechanism of this reaction, but its 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°C 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 $-N^+(CH_3)_3$ 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 sphingomyelinases 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. Nevertheless, it does not necessarily cause damage in the endocarditis of rabbit and pneumonia models (Salgado, 2014).

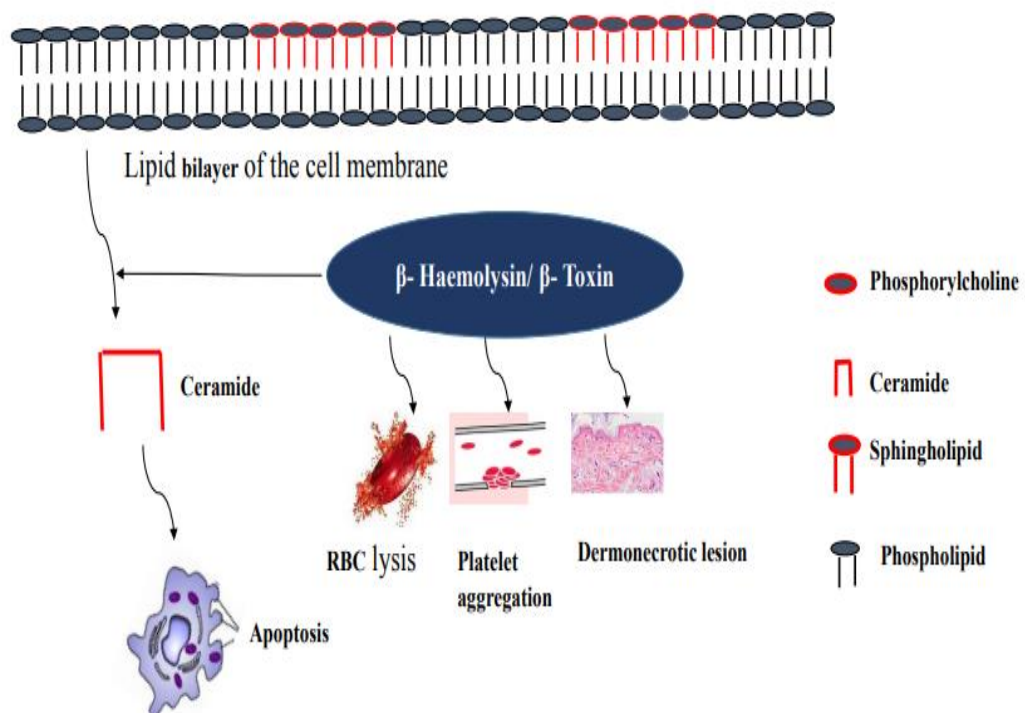


Figure 4. β-toxin activity on the cell membrane (Sireesha et al., 2019).

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 encoding 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 neutrofiles (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 bacteria 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 <i>et al.</i> , 1997)
Fibrinogen binding protein	SE0331	sdrG(fbe)	Fibrinogen binding protein (Nilsson <i>et al.</i> , 1998; Davis <i>et al.</i> , 2001)
SD-rich cell surface adhesin	SE2395, SE1632	sdrF,sdrH	Not known (McCrea <i>et al.</i> , 2000)
Extracellular matrix binding protein Embp	SE1128	embp	Fibronectin binding protein (Williams <i>et al.</i> , 2002)
Elastin-binding protein	SE1169	ebpS	Adhesion on host proteins (Park <i>et al.</i> , 1996)
Cell accumulation	SE0175	aap	Accumulation associated protein (Accession No: AJ249487)
Fmt	SE0754	fmt	Autolysis and penicillin resistant (Komatsuzawa <i>et al.</i> , 1997)
Exoenzymes			
Serine protease, V8 protease	SE1543	sspA	Degrade or digest proteins (Rice <i>et al.</i> , 2001)
Glycerol ester hydrolase	SE2403	geh	Degrade lipids (Simons <i>et al.</i> , 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
Metalloprotease	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
Exonuclease	SE1028, SE1029		Possible degrade host nucleic acid
Toxins			
Delta-haemolysin	SE1634	hld	Destruction of blood and tissue cells (McKevitt <i>et al.</i> , 1990)
Beta-haemolysin	SE0008		Phospholipase C synthesis (Katerov <i>et al.</i> , 1994)
Others			
67 KDa myosin cross-reactive protein	SE0776		Cross-reactive with host cardiac myosin (Dale and Beachey, 1985)
Putative lipoprotein similar to streptococcal PsaA	SE0405		Putative adhesin essential for virulence (Berry and Paton, 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* encourage 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 endurance 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) separation and dispersion of planktonic cells (Costerton et al., 1999; Sutherland, 2001; Flemming, 2010).

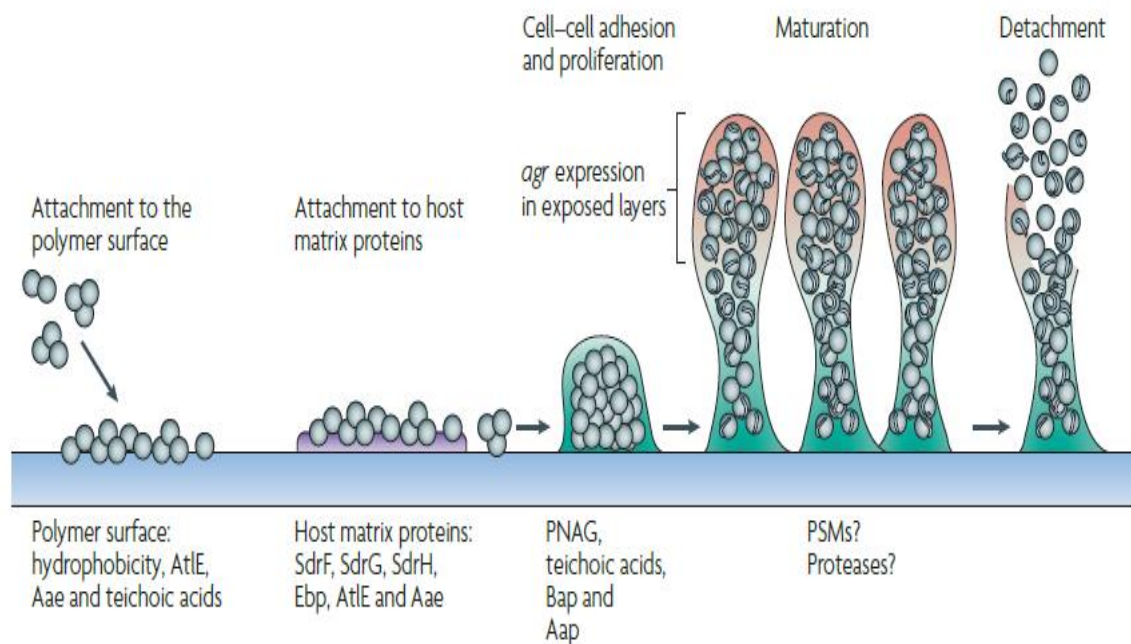


Figure 6. Bio-film formation of *S. epidermidis* in relation to the virulence factors in each phase (Otto, 2009).

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).

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

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; PSM α , PSM β and PSM γ 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 pathogenicity 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), Staphylococcal cassette chromosome *mec* (*Scmec*). 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 *SCCmec* structures are identified in *S. epidermidis*. The most abundant form was *SCCmec* 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° C with glycerol (15% v/v) at REDPROM laboratory, Aydın 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 121⁰ 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 121⁰ 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 121⁰ C for 15 minutes. After cooling to 45⁰ 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 121⁰C for 15 minutes. The media was allowed to cool to 45⁰C -50⁰C. 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 +4⁰ 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°C. 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°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°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 transferred into eppendorf tubes containing 1 mL distilled water and stirred to homogeneity.
- The eppendorf tubes were centrifuged for 5 mins at 11000 rpm
- The supernatants were discarded
- 100 µL of Lysis solution were added and pipetted to homogeneity
- The tubes were incubated at 56°C for 30 mins and a subsequent reincubation at 100°C for 10 mins
- The tubes were vortexed and stored at -20°C until use.

3.2.2. PCR primer sequence design

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

```
1  atgaaacgag gtgtaacaat attgaattgg caacgtaaatt gtataactaac tactttgttg
61  gttttaagta gtttattttt agtatttttc actatcacat atgcgagtga acgtgatttt
121 aaagacagtc ttaaaatcac tacacacaaac gtgtatttct tacctactgc tatctaccct
181 aattggggac aatctcagcg cgctgattta atttcaaaag cagattacat tcaaaatcaa
241 gatgtcgtga ttctaaatga attatttgat aaaaaagctt ctaaaagatt gttaacacgt
301 ctacattcac agtaccctta tcaaacacct atcgttggta aggttacaga aggttggcaa
361 aatacttctg gtacttatag aaaaattaaa aaagtaagtg gtggcgttgg tattgtgagt
421 aaatggccta tcgtacaaca agaacaacat atttataaaa aaggttgagg ggctgatatg
481 gcaggaataa aaggctttgc ctacattaaa attaataaga atggcaataa ccaccatatt
541 atcggaacac atctacaagc tgaagatcca acatgcttta aaggaaaaga taaggacatt
601 agacagagtc aaatgagtga aattaaacag tttatcaaag acaagaatat ccctaaaaat
661 gaaccggtct atatcggttg tgacttaaat gtcattaaag attcagatga atatcaacaa
721 atggcaataa acttaaatgt ttcattacct actcaattcg atggtaaatgc atatagttgg
781 gatactagca gtaatatgat tgcgaaatat aattatccta aattagaacc tcaacactta
841 gattatattt tattagatcg tgaccatgca caaccaagct catggcataa tgatacacat
901 agagtgaagt caccagaatg gtccgtgaaa tcttggggta aaacatacaa atacaatgat
961 tactcagatc attaccact ctcgtgctat 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 *KpnI* and *EcoRI* restriction sites using comparative tools such as NCBI, Reverse compliment, Multiple Primer Analyser and Ncbutter.

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

Fwd KpnI-HIb	ctaGGTACC atgaaacgag gtgtaacaat
Rev EcoRI-HIb	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	Initial 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 -*pst*I 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 (*hly*) gene

The amplicon products and the plasmid (pUC19) were cut by restriction enzymes (*KpnI* 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
<i>KpnI</i> restriction enzyme	1 µL	1 µL
<i>EcoRI</i> 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 eppendorf tubes and incubated at 37°C 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 dH₂O 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°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 eppendorf tubes. The eppendorf tubes were incubated with a thermo shaker (MS-100) for 1 hr at 22°C, after which it stayed overnight at 16°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 37⁰ 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 +4⁰ C for 10 mins. The supernatant was decanted, pellet was resuspended with dH₂O and then centrifuge at >5000 rpm at +4⁰ 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 +4⁰ C. The supernatant was decanted, re-suspended with 30 mL of cold solution mixture of (80mL MgCl₂ and 20mL CaCl₂) and centrifuge at >5000 rpm for 10 mins. The supernatant was decanted and 1mL of 0.1M CaCl₂ was added and mixed gently to homogeneity (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°C 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° 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 37⁰ C. After the growth, at approximately 15 white colonies (i.e. transformants) from the experimental 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°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 **M13R**_ 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°C for 24 hrs (Pulicherla et al., 2013). After incubation, plasmids from bacterial cultures were isolated by miniprep plasmid isolation kit (Geneaid Presto™ Mini Plasmid Kit) according to the manufacturers protocol:

- From the overnight incubated bacterial culture, 1.5 mL was transferred 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 μ L 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 μ L 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 μ L 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 μ L 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, *KpnI* and *EcoRI* restriction enzymes were used for the cutting. For the final confirmation, 5 μ L 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 -20⁰C.

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 37⁰ C for 24 hours. The other plate was incubated at 37⁰ C for 18 hours was then transferred to a lower temperature of +4⁰ 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.

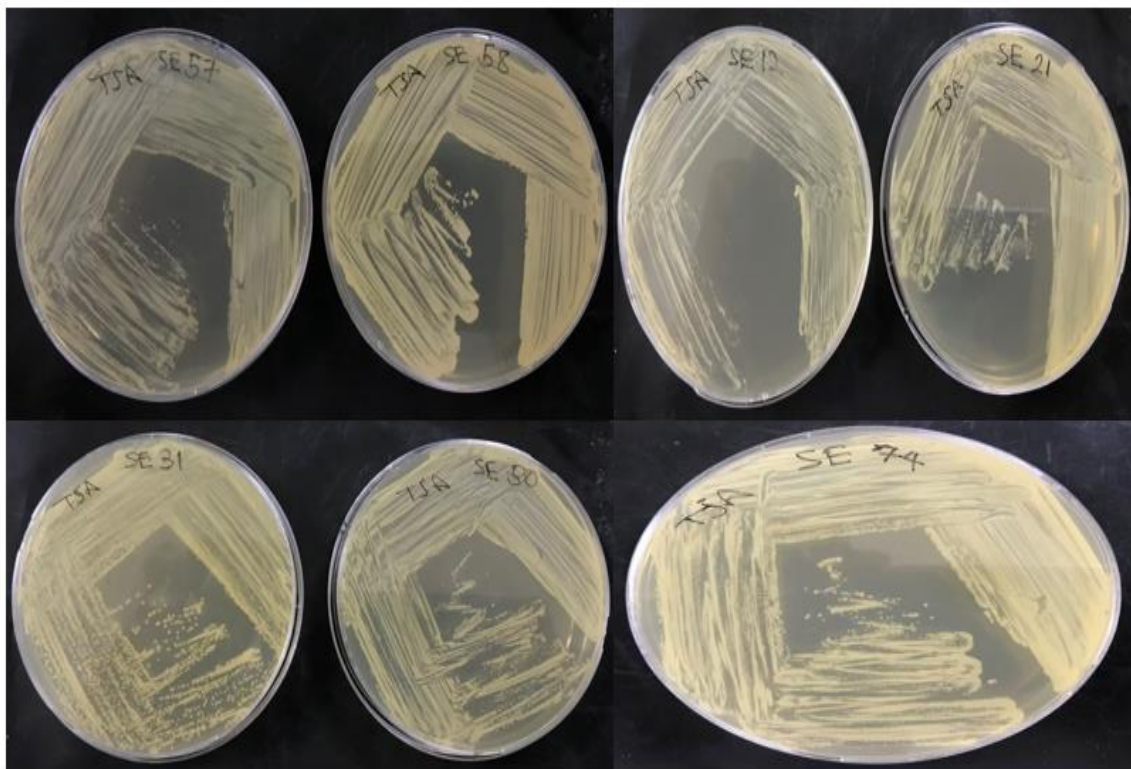


4. RESULTS

Beta hemolysin gene (*hly*) represent one of the most studied and important virulence factors in the pathogenicity of Coagulase-negative Staphylococci. The *hly* gene used in this study was detected among CoNS strains in our laboratory collection, and amplified by using PCR. The amplified *hly* 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

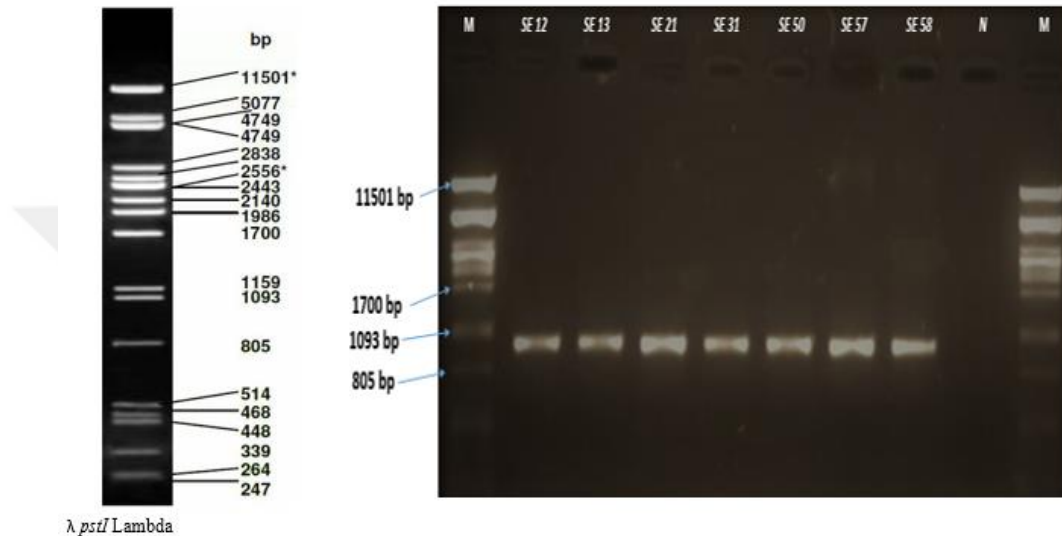
S. epidermidis is a Gram-positive, cocci- shaped and coagulase negative bacterium. The isolates were grown on Tryptic Soy Agar (TSA) plates as shown in (Picture 1).



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

4.2.PCR amplification of beta hemolysin gene

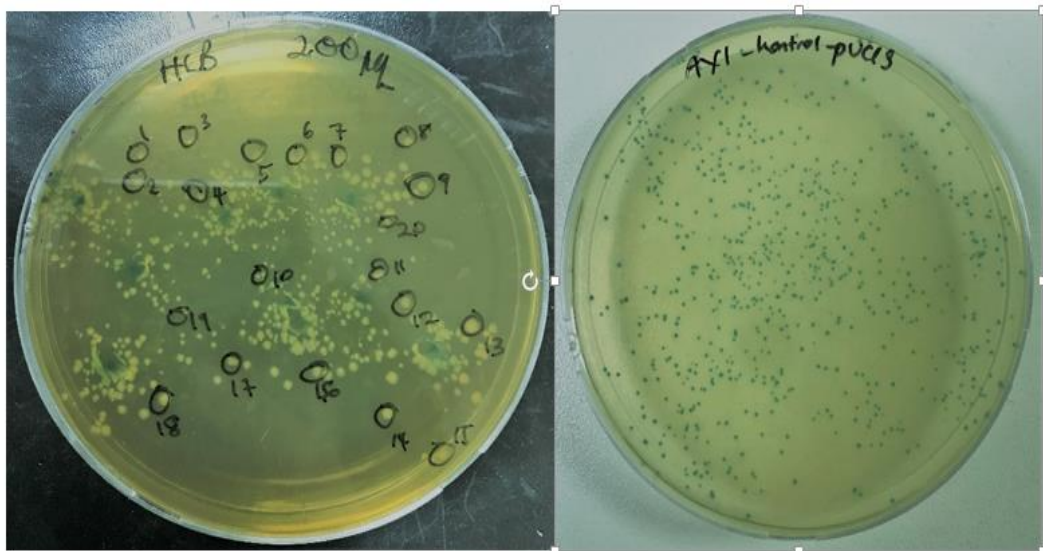
The (Picture 2) below, is a (1.0%) agarose gel resolving PCR products generated for cloning. The expected amplicon size was **1005 bp**. From the 7 strains of *S. epidermidis* (SE12, SE13, SE21, SE31, SE50, SE57 and SE58), the PCR result showed positive for all the strains, with N as the negative control.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

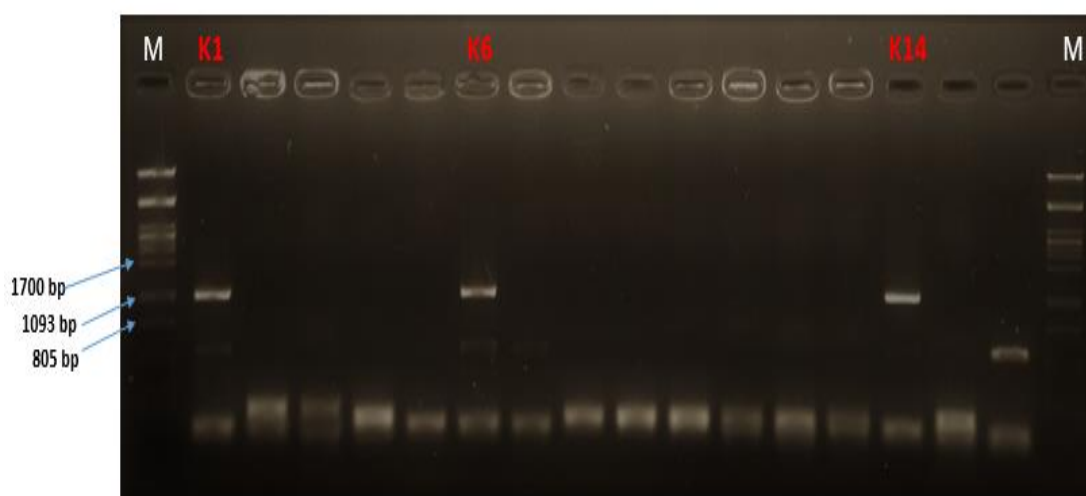


Picture 3. Blue and White colonies after transformation.

(A) White colonies are the colonies with recombinant plasmid. (B) Control plate with transformed pUC19 plasmid (pUC19) vector only.

4.4. M13 colony PCR

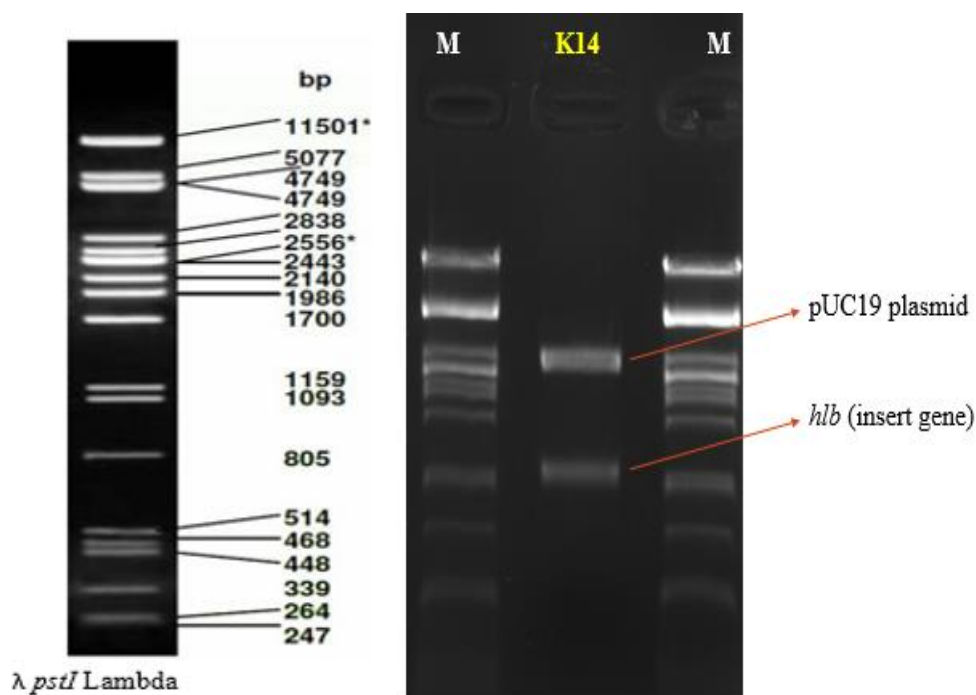
Colony PCR based strategy was performed to determine clones that harboured pUC19 plasmid with the desired gene insert cloned into the Multiple Cloning Site (MCS). From the PCR result obtained, clones (K1, K6 and K14) carried positive band with the exact size (1005 bp) as expected in the (**Picture 4**)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



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

4.5. Plasmid isolation from recombinant colonies

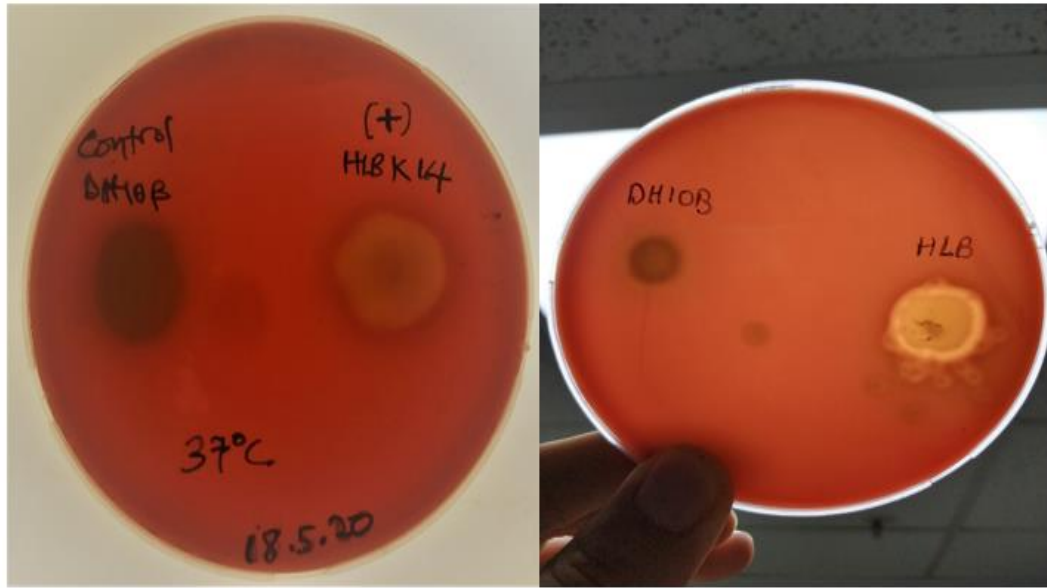
The purified plasmid was cut with *KpnI* and *EcoRI* restriction enzymes and electrophoresed for confirmation. After electrophoresis, two different bands were confirmed, one indicating the insert gene with the other band representing the vector plasmid (pUC19).



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 (*hly* gene). M: (λ -*pstI*) 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 recombinant beta hemolysin on sheep blood agar. Hemolysis of erythrocytes was confirmed positive (+) for the clone 14 and negative (-) 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 alignment 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.

Descriptions

Graphic Summary

Alignments

Taxonomy

Sequences producing significant alignments

Download

New

Select columns

Show

select all

73 sequences selected

GenBank

Graphics

Distance tree of results

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. L
☒	Staphylococcus epidermidis strain Q47 chromosome, complete genome	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	25181
☒	Staphylococcus epidermidis strain none genome assembly, chromosome: 1	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	25338
☒	Staphylococcus epidermidis strain IRL01 chromosome, complete genome	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	27374
☒	Staphylococcus epidermidis strain NCTC13924 genome assembly, chromosome: 1	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	27508
☒	Staphylococcus epidermidis strain DAR1907 chromosome, complete genome	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	27275
☒	Staphylococcus epidermidis isolate BPH0662 genome assembly, chromosome: 1	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	27930
☒	Staphylococcus epidermidis strain Z0118SE0260 chromosome, complete genome	Staphylococcus epidermidis	1530	1530	98%	0.0	98.95%	25100
☒	Staphylococcus epidermidis strain none genome assembly, chromosome: 1	Staphylococcus epidermidis	1524	1524	98%	0.0	98.83%	25565

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

Download ▾ GenBank Graphics

Staphylococcus epidermidis strain O47 chromosome, complete genome

Sequence ID: CP040883.1 Length: 2518181 Number of Matches: 1

Range 1: 13236 to 14070 GenBank Graphics ▾ Next Match ▴ Previous Match

Score	Expect	Identities	Gaps	Strand
1495 bits(809)	0.0	829/838(99%)	3/838(0%)	Plus/Plus
Query 14	CTGAGTAATCATTGTATTTGTATGTTTTCCCGGGATTTTACTGGACCACTCTCTGATG	73		
Sbjct 13236	CTGAGTAATCATTGTATTTGTATGTTTTCCCAAGATTTTAC-GGACCA-T-TCTGGTG	13292		
Query 74	ACTTCACTCTATGTGTATCATTATGCCATGAGCTTGGTTGTGCGTGGTCACGATCTAATA	133		
Sbjct 13293	ACTTCACTCTATGTGTATCATTATGCCATGAGCTTGGTTGTGCGTGGTCACGATCTAATA	13352		
Query 134	AAATATAATCTAAGTGTTGAGGTTCTAATTTAGGATAAATTATATTTTCGCAATACTATTAC	193		
Sbjct 13353	AAATATAATCTAAGTGTTGAGGTTCTAATTTAGGATAAATTATATTTTCGCAATACTATTAC	13412		
Query 194	TGCTAGTATCCCAACTATATGCATTACCATCGAATTGAGTAGGTAATGAAACATTTAAGT	253		
Sbjct 13413	TGCTAGTATCCCAACTATATGCATTACCATCGAATTGAGTAGGTAATGAAACATTTAAGT	13472		
Query 254	TATTTGCCATTTGTTGATATTCATCTGAATCTTTAATGACATTTAAGTCACCCCGATAT	313		
Sbjct 13473	TATTTGCCATTTGTTGATATTCATCTGAATCTTTAATGACATTTAAGTCACCCCGATAT	13532		
Query 314	ACACGGGTTTCATTTTATAGGGATATTCTTGCTTTGATAAACTGTTTAATTTCAATTCATTT	373		

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 its 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 pathogenicity of *S. epidermidis* is very essential due to its 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 (*hly*) 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 dysfunction and immunomodulation (Singh et al., 2014).

Our present study was aimed to clone and sequence recombinant *hly* gene from the isolates of *S. epidermidis* by testing its potential efficacy of hemolysis on sheep blood agar. The *hly* 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 37⁰ C, followed by a subsequent temperature at 4⁰ 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 (*hly*) gene has being performed on various dimensions. In our study; the presence of *hly* gene was positive among the *S. epidermidis* isolates tested. In the study of (Nasaj et al., 2020), *hly* gene was screen among 91 CoNS isolates from various clinical specimens. Their results showed the presence of *hly* 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°C for 18 hrs, and a subsequent reincubation at 4°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).

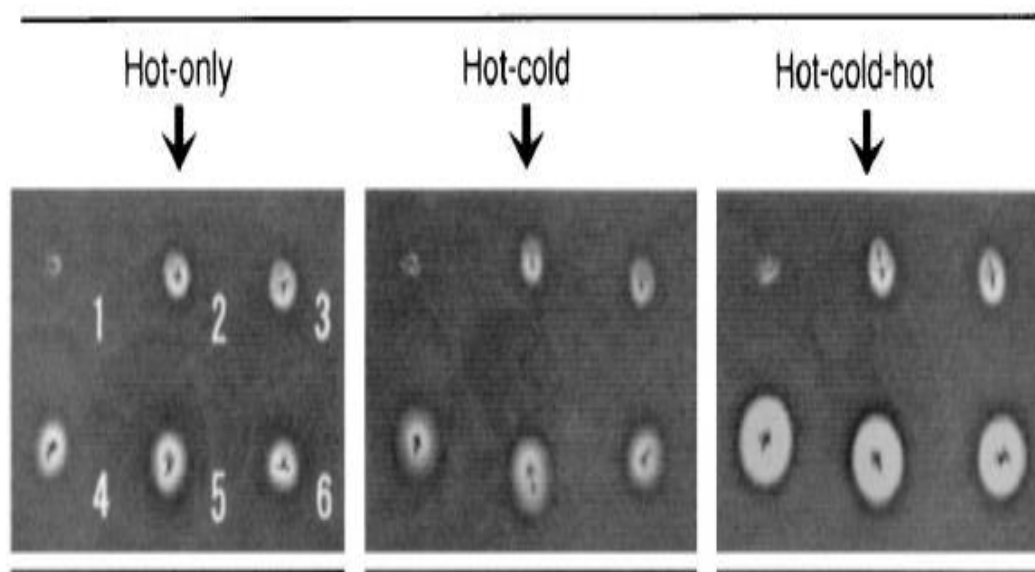


Figure 11. Hemolytic zones exhibited by six GBS on erythrocytes.

At the Hot-only; the plates were incubated at 37⁰ C for 24 hrs. For the Hot-cold, the plates were incubated at 37⁰ C for 18 hrs, followed by a reincubation at 4⁰ C for 6 hrs. Finally at the Hot-cold-hot, the plates were incubated at 37⁰ C for 18 hrs, followed by incubation at 4⁰ C for 6 hrs and a subsequent reincubation at 37⁰ 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 (*hly*) 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 experiments, 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.

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