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Hemolysins of *Staphylococcus aureus*—An Update on Their Biology, Role in Pathogenesis and as Targets for Anti-Virulence Therapy

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Abstract

Staphylococcus aureus is a dangerous gram positive bacterial pathogen which, not only evades the host's immune system but also can destroy the leucocytes especially neutrophils. It has an embodiment of virulence factors most of which are secreted. *Staphylococcus aureus* secretes a number of toxins which cause tissue damage and facilitate spreading and nutrients uptake. Among the toxins, hemolysins α, β, γ, δ and Panton Valentine Leukocidin (PVL) are unique that they drill pores in the membrane, leading to the efflux of vital molecules and metabolites. Hemolysins also help in the scavenging of iron, although many of them also have leucolytic properties. α-hemolysin, also known as α-toxin, is the most prominent cytotoxin which damages a wide range of host cells including epithelial cells, endothelial cells, erythrocytes, monocytes, keratinocytes and it damages cell membrane and induces apoptosis. β-Hemolysin significantly affects human immune cell function. It has Mg^{2+} dependent sphingomyelinase activity and degrades sphingomyelin of plasma membrane into phosphorylcholine and ceramides. The bi-component leukocidins, which include γ-hemolysin and PVL, attack human phagocytic cells and greatly contribute to immune evasion. Delta toxin is a low molecular weight exotoxin with a broad cytolytic activity. Virulence determinants, quorum sensing and biofilm synthesis provide some attractive targets for design and development of a new group of antimicrobial compounds. This review provides an update on the structure, biological functions of hemolysins and their role in quorum sensing/biofilm synthesis (if any) and as effective therapeutic targets for anti-virulence drug development. We have tried to bring

together information available on various aspects of hemolysins and highlighted their distribution among all species of Staphylococcus and other bacteria. We have updated the status of development of candidate drugs targeting the hemolysins for anti-virulence therapy as it offers an additional strategy to reduce the severity of infection and which would, through quorum quenching, delay the development biofilms leading to drug resistance.

Keywords

Staphylococcus aureus, Hemolysins, PVL, Quorum Sensing, Biofilm, Anti-Virulence Therapy

1. Introduction

S. aureus, which was first discovered by Sir Alexander Ogston in 1880 [1], has long been recognized as a serious threat to human health, capable of causing a multitude of infections. It is a commensal bacterium which doubles-up as one of the most common human pathogens causing both nosocomial and community-acquired infections. It produces a variety of virulence factors depending on the site of infection ranging from minor skin infections to life-threatening diseases such as osteomyelitis, endocarditis, skin and soft tissue infection, brain abscesses, meningitis, bacteremia and pneumonia [1].

A major pathogenic attribute of *S. aureus* is to escape both innate and adaptive immune responses wherein a number of virulence factors including surface components such as the capsule, coagulase, protein A, teichoic acid, polysaccharides and adhesins; enzymes such as esterases, lipases, fatty-acid modifying enzymes, various proteases, hydrolytic enzymes, catalase, β-lactamase and various toxins such as leukocidin, enterotoxins, TSST-1 and alpha, beta, gamma and delta hemolysins [2], play important roles. Interestingly, several of these secreted exotoxins are directed to damage the host cells plasma membrane. Of the wide variety of *Staphylococcal*cytolytic exotoxins produced, the most prominent and well-characterized ones are the hemolysins, which play an important role in the *Staphylococcal* disease pathogenesis and have the ability to kill a variety of host cell populations including immune cells and help the bacteria to spread within the host [3]. The first report of hemolytic activity of *Staphylococci* recovered from human lesions appeared in 1894 and subsequently in 1900 [4].

Virulence determinants, quorum sensing and biofilm synthesis provide some attractive targets for the design and development of a new group of antimicrobial compounds. This review provides an update on the structure, biological functions of hemolysins and their role in quorum sensing/biofilm synthesis (if any) and as effective therapeutic targets for anti-virulence drug development. We have tried to bring together information available on various aspects of hemolysins and highlighted their distribution among all species of Staphylococcus and other bacteria. We have updated the status of development of candidate drugs

targeting the hemolysins for anti-virulence therapy as it offers an additional strategy to reduce the severity of infection and which would, through quorum quenching, delay the development biofilms leading to drug resistance.

Alpha toxins are especially hemolytic to rabbit erythrocytes though they act on a wide range of mammalian cells including human erythrocytes. This toxin is a heptamer, toxic to epithelial cells, dermonecrotic and neurotoxin in human host [5]. Currently, pore formation by α-toxin at the molecular level is being investigated. **β-toxin** is magnesium-dependent sphingomyelinase that is active on sheep erythrocytes [6]. **γ-toxin** is a two-component exotoxin which comprises of six different combinations of proteins active against erythrocytes [5]. **δ-toxin** is a low molecular weight toxin with the ability to lyse many cell types [7]. Generally, *S. aureus* infection is multifactorial due to the combined action of several virulence determinants. One exception is toxinoses, which are caused by toxic shock syndrome toxin, exfoliative toxins A&B and different *Staphylococcal* enterotoxins [8].

2. Virulence Factors

Virulence factors play several roles in the host including invasion, mediating immune suppression, and degrading host cells or tissues to obtain space for spreading or to acquire nutrients for bacterial growth [5]. Several factors contribute to *S. aureus* pathogenicity, like the surface proteins which mediate bacterial adherence to host cells, secretion of series of extracellular toxins, and enzymes which destroy the host's cells and tissues, help the bacteria to escape from host immune defense, growth and spread of bacteria in the host [8]. Extracellular toxins are proteins secreted by *S. aureus* during the post exponential and early stationary phases. *In vitro* and *in vivo* studies in animals revealed that Pore Forming Toxins (PFT) are major virulence factors involved in the pathophysiology of *Staphylococcal* infections. These toxins are capable of targeting a wide variety of immune cells during infection, such as human polymorphonuclear leukocytes, monocytes and macrophages and help the bacteria to escape from both innate and adoptive immune responses [8]. Hemolysins are not only present in staphylococcal species (**Table 1**) but also in several other pathogenic bacteria (**Table 2** and **Figure 1**).

Genome analyses revealed that there are different sets of genes encoding different virulence factors in *S. aureus*. This heterogeneity is due to the fact that many of these genes are located in pathogenicity islands wherein genes are translocated by Horizontal Genome Transfer (HGT) via phage transduction, conjugation or by direct uptake of naked DNA by genetic competence [27].

2.1. Alpha Hemolysin (Alpha-Toxin, Hla)

2.1.1. Discovery

Initial studies on investigation of the toxic activity of staphylococcal supernatants began in the late 1800s. These toxic substances secreted by *S. aureus* were attributed lethality in guinea pigs and rabbits, dermonecrosis, inflammation of

the conjunctival epithelium and hemolysis [28]. Investigation of *α*-toxin arose by a tragedy in the Australian town of Bundaberg in 1928. Twenty one children who were vaccinated with a diphtheria toxoid became severely ill and 12 died. Investigating the cause of this disaster, Burnet reported that the toxic properties of crude exo-substance from *S. aureus* led to the identification of *α*-toxin as a major cause of the observed toxicity [29].

Table 1. Hemolysins from *Staphylococcal* species.

S. No	*Staphylococcal* species	Type of hemolysin	Disease pathology	Ref
1	*Staphylococcus aureus*	*α, β, γ, δ*	Sepsis	[9]
2	*Staphylococcus auricularis*	*α*	Weak or Moderate hemolysins associated with sepsis	[10]
3	*Staphylococcus carnosus*	Lack of hemolysins	-	[11]
4	*Staphylococcus epidermidis*	*α, δ*	CoNS species, plays a role in catheter-associated blood stream infections	[12]
5	*Staphylococcus haemolyticus*	*α, δ*	CoNS, associated with blood stream infections in Neonates	[10]
6	*Staphylococcus hyicusintermedius*	*δ*	Associated with bovine mastitis	[13]
7	*Staphylococcus lugdunensis*	*δ*	Common cause of Skin and soft tissue infections	[14]
8	*Staphylococcus saprophyticus*	*α*	Associated with uncomplicated urinary tract infections	[15] [16]
9	*Staphylococcus sciuri*	*β, δ*	Associated with mastitis in animal infections. In humans, it causes wound and soft tissue infections, abscess, boils, peritonitis, and endocarditis	[17]
10	*Staphylococcus simulans*	*δ*	Urinary tract & wound infections	[18]
11	*Staphylococcus warneri*	*δ*	Septic arthritis	[10]

Table 2. Hemolysins from different bacterial species.

S. No	Organism	Type of Hemolysins	Salient features	Ref
1	*Escherichia coli*	hlyA (*α*-hln)	Forms pores on the host cell membrane; potentially toxic to monocytes, lymphocytes and macrophages	[19]
2	*Serratia marcescens*	shlA	Cytotoxin, acts on epithelial cells (pore formation) causes ATP depletion and potassium efflux	[20]

Continued

3	*Aeromonas hydrophila*	*β*-hln	Epithelial barrier impairment	[21]
4	*Listeria monocytogenes*	Listeriolysin-O (LLO)	Pore forming cholesterol dependent cytolysin, lysis of the phagosome	[22]
5	*Streptococcus pneumoniae*	*A*-hln/pneumolysin	Membrane pore forming toxin-causes inflammation in meningeal membrane leads to meningitis	[23]
6	*Plasmodium*	*Plasmodium* hemolysin III (PfhlyIII)	Lysis of erythrocytes leading to hemolytic anemia	[24]
7	*Leptospirasps*	*β* -hln sphingomyelinase	Phospholipases act on erythrocytes and other cell membranes containing phospholipid as a substrate-causes hemolysis and hematuria	[25]
8	*Bacillus cereus*	*Bacillus cereus* Sphingomyelinase (Bc-SMase)	Lysis of erythrocytes which contains sphingomyelin	[26]

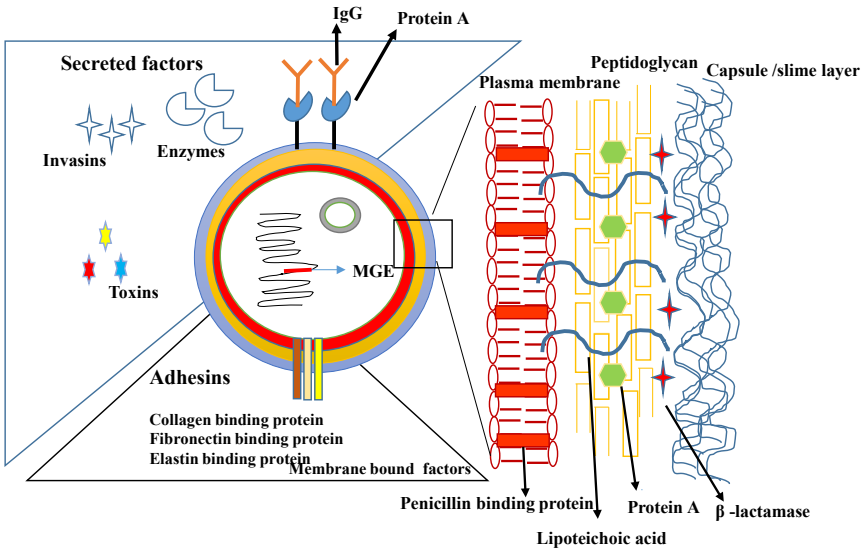

Figure 1. Virulence factors in *S. aureus.*

2.1.2. Chemical Nature of Alpha-Toxin

The alpha (*α*) gene/toxin is located on the bacterial chromosome [30] and is one of the best characterized pore forming toxin encoded by the *hla*. It plays an important role in *S. aureus* infections including pneumonia, skin and soft tissue infections [SSTI], sepsis, septic arthritis, brain abscess and corneal infections [31] [32] [33] [34] [35]. The exotoxin is a secreted protein of 293 amino acids (no cysteine) and has pI of about 8.5, endowed with hemolytic, cytotoxic, dermonecrotic and lethal properties. Circular Dichroism studies discovered that the mature toxin is composed almost entirely of *β*-strands [36]. This 33 kDa pore forming toxin is expressed as a monomer by almost all the clinical isolates of *S.*

aureus that assemble to form a membrane-bound heptameric β-barrel pore (232.4 kD).

Though α-toxin is active against a wide range of mammalian cells, it displays species and cell type specificity. It is predominantly active against rabbit erythrocytes when compared to human erythrocytes and other mammals and this toxin at, as low as 1 μg is lethal when injected into rabbits intravenously. Human lymphocytes and monocytes are α-hemolysin susceptible (maximum lysis at 100 $ng \cdot ml^{-1}$) whereas granulocytes are highly resistant to α-hemolysin (no lysis even at 10 $\mu g \cdot ml^{-1}$) [37]. Lysis of human lung epithelial cells (A549) is observed at high α-hemolysin concentrations (30 $\mu g \cdot ml^{-1}$) [38].

2.1.3. Structure of α-Hemolysin

Structure of α-hemolysin protein has been resolved by X-ray crystallography. It is expressed as a water-soluble monomer (33.2 kDa) which assembles to form a membrane-bound heptameric pore on the susceptible cells. It is a mushroom-shaped protein and is divided into three sub-domains (**Figure 2**).

1) **Cap domain**: It is the external part and is largely hydrophobic in nature and is essential for the entry into the pore; each monomer contains two six stranded anti-parallel β-sheets which are exposed to the extra-cellular medium.
2) **Rim domain**: It is the base of the cap, in close proximity to membrane bilayer; charged amino acid residues at the lateral surfaces of the rim may provide contact sites with polar head groups of lipids in host cell membranes. Several aromatic amino acids (tryptophan, tyrosine) at the base of the rim may provide the contact sites for the interaction of pre-pore with the hydrophobic lipid environment of the plasma membrane
3) **Stem/Trans membrane domain**: This is largely hydrophilic in nature, forming the membrane-perforating β-barrel pore [31]. The residues 118-140 in the sequence of alpha-hemolysin or α-toxin (Hla) monomer form the main portion of the pre-stem domain. Replacement of histidine residue at 35 positions by leucine completely gets the transition of alpha-hemolysin or α-toxin (Hla) heptamer from the pre-pore to the pore formation stage [38].

2.1.4. Mode of Action

The lethal properties of α-toxin are probably due to its effect on cells involved in the maintenance of homeostasis. Pore formation is a multi-step process; which includes secretion of α-hemolysin monomers, which bind to target membranes, oligomerize to form a functional heptameric pore complex, the pre-pore. Susceptible cells have specific receptors which allow the alpha toxin to bind, causing small pores of 14 Å resulting in the leakage of small cytoplasmic contents less than 2 kDa, like Ca^{2+}, K^{+}, altering the ion gradients and eventually causing death of the cell [28]. The pore concept has been widely accepted (**Figure 3**).

Interaction of alpha-hemolysin or Hla with host cells is concentration dependent. At higher concentration of α-hemolysin, pore formation is recep-

tor-independent and is likely mediated by the interaction between α-hemolysin and phosphocholine head groups but at lower concentration pore formation is dependent on the interaction of receptor. Pore formation and subsequent cell lysis do not occur at concentrations below 33 ng/μL [39]. However, the recent findings suggest that α-hemolysin associates with a receptor protein ADAM10 (A Disintegrin and Metalloprotease 10). ADAM10 is a zinc-dependent metalloprotease that is expressed on the surface of many distinct cell types as a type 1 trans-membrane protein. Upon binding of α-hemolysin with ADAM10, the complex re-localizes to caveolin-1enriched lipid rafts that serve as a platform for the series of intracellular signal events and this interaction was diminished upon si-RNA mediated knockdown of ADAM10 [38]. α-hemolysin binding to different cell types like RBC and human epithelial cells correlates with ADAM10 expression. Sequential binding to these different receptors (ADAM10 & Caveolin) might trigger a conformational change allowing α-hemolysin oligomerization to initiate cell specific lysis.

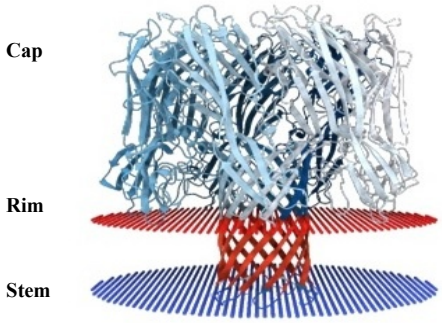

Figure 2. Structure of α-hemolysin (PDB ID: 7 AHL).

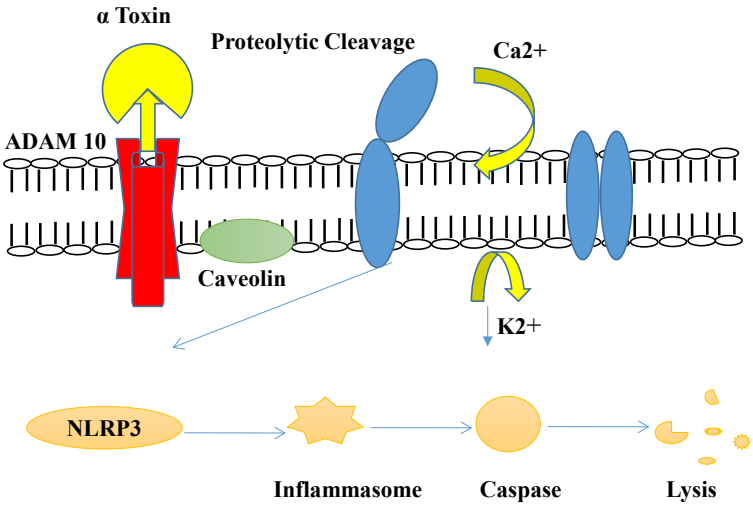

Figure 3. Mechanism of Action of alpha hemolysin: 1) α-toxin binds to cell membrane receptor ADAM10. 2) Rise in Ca^{2+} and rapid fall in K^{+}. 3) Activation of NLRP3 inflammasome. 4) Caspase-1 activation which leads to cell lysis [31].

2.1.5. Pathophysiology

The pore formation actually depends on the concentrations of alpha-hemolysin or α-toxin (Hla) monomers in the medium and the presence of receptors in cell membrane. Experimental evidence indicates that only one or two potential receptors for Hla monomers exist in mammalian cells, *i.e.* ADAM10 and alpha-5-beta-1integrin [38] [40] and it is not known whether there are other receptors. Interaction of α-hemolysin with ADAM10 ultimately leads to the disruption of focal adhesions by dephosphorylation of structural proteins like FAK (Focal Adhesion Kinase), paxillin, Src (Tyrosine protein kinase), and p130Cas (involved in cellular adhesion) which provide tensile strength to the barrier [38]. Alpha toxin induces the release of cytokines and chemokines. Excessive production of interleukin-1β and TNF-α results in tissue injury during inflammation. This toxin also induces apoptosis in human T-cell and monocytes. Neurotoxicity by α-toxin was observed due to the destruction of myelin in rabbit nerves and in the murine cerebral cortex [28]. Low doses of the toxin enhance phagocytosis and the intracellular killing of neutrophils.

Production of pore forming toxins PVL and Hla facilitates the pathogenesis of pneumonia in *S. aureus*/MRSA infections and an increase in the incidence of severe pneumonia [41]. Immunization of mice with inactive α-toxin protected mice against *S. aureus* pneumonia [32]. Hla mutants of *S. aureus* display reduced virulence in invasive disease models of pneumonia [32] [41] dermonecrotic skin infection, sepsis, peritonitis, and infection of the cornea [34], central nervous system [33], endocardium, and the mammary gland. However when the mutated α-toxin gene in the knock out strain was complemented, the pathology was restored. *S. aureus* (USA300) displays increased *hla* expression and virulence in experimental models by accessory gene regulator (*agr*) and staphylococcal accessory element (*sae*) regulatory system which controls the toxin expression. *In vivo* studies revealed that alpha-hemolysin could be a useful target in the treatment of staphylococcal mastitis.

Moreover α-toxin has the ability to alter the platelet morphology and hence may influence the thrombotic events associated with *S. aureus* sepsis [42].

Most of the staphylococcal exo-proteins are synthesized at post-logarithmic phase under the control of *agr* (accessory gene regulator). The actual effector of this global regulatory system is RNAIII which stimulates the transcription of genes coding for exo-proteins. Expression of α-toxin is also controlled by a two-component system named as Agr system which was first identified by Recsei*etal* [43], expression can also be modulated by other regulators, such as *S. aureus*exo-protein Response regulator (*SaeR*), *Staphylococcal* accessory regulator Z (*SarZ*), signal transduction histidine protein kinase (*ArlS*) (up-regulators) and Repressor protein gene (*Rot*), *Staphylococcal* accessory regulator (*SarT*) (down-regulators).

2.1.6. As a Drug Target

Alpha hemolysin, has received significant attention as a target for anti-toxin

neutralizing antibodies. Anti-*α*-hemolysin antibodies provide protection against lethal staphylococcal pneumonia caused by *S. aureus* in experimental studies [44] [45] and significantly reduced abscess formation in an *S. aureus* dermonecrotic model [46] [47]. Moreover, the efficiency of monoclonal antibodies seems to be additive or synergistic when administered along with clinically used antibiotics (vancomycin or linezolid) [45] [47], which suggests that the treatment by antibiotics along with antibodies will be more efficacious than either of them. A number of compounds or molecules which block the *α*-hemolytic activity of *S. aureus* hemolysin have been discovered. Recent studies revealed that *β*-cyclodextrin derivative inhibits the *α*-hemolysis *in vitro* and protects the host against *S. aureus* infection [48] [49] *Insilico* tools and simulation programs have enabled to find other inhibitors for *α*-hemolysin [50] [51] [52] [53]. High throughput virtual screening of peptide or compound libraries facilitated to find potent inhibitors for the crystal structure of *α*-hemolysin [54] [55]. The host receptor for *α*-hemolysin, apparently the ADAM10, is an attractive target for the design of an inhibitor to block the toxin receptor interaction [56] and to reduce the lesions and the severity of recurrent skin and soft tissue infection [57] (**Table 3**).

2.2. Beta Hemolysin

2.2.1. Discovery

β-toxin is a non-pore forming hemolysin, a neutral sphingomyelinase secreted by the majority of *S. aureus* isolates and it was first identified by Glenny and Stevens in 1935 [60]. A 4 kb *ClaI*chromosomal fragment which encodes 330 amino acid polypeptide with an approximate molecular weight of 39,000 Daltons. A 200 residue region of homology (55.7%) has been found with *Bacillus cereus* sphingomyelinase [61]. The N-terminal sequence of secreted *β*-hemolysin is GluSerLysAspAspThrAspLeuLys corresponding to residues 35 - 44 [62]. Initial 34 residues of the polypeptide contain a signal sequence which gets cleaved upon secretion, resulting in a mature protein with a molecular weight 35,000. Some studies have shown that *β*-hemolysin is extremely basic, with an isoelectric point above pH 9. *β*-toxin lyses erythrocytes mainly for its source of haem. It is highly active against sheep erythrocytes but not on rabbit erythrocytes [6].

2.2.2. Chemical Nature of *β*-Hemolysin

It is also called as hot-cold hemolysin due to the enhanced haemolytic activity observed below 10˚C after incubation at 37˚C overnight. It is also known as sphingomyelinase. So far three forms of sphingomyelinases have been identified based on the isoelectric pH. One of them is acidic, the second one is neutral and the third one is alkaline. *Staphylococcus aureus* beta toxin is an Mg^{2+} dependent neutral sphingomyelinase that hydrolyzes sphingomyelin into phosphorylcholine and ceramide [N-acyl sphingosine]. The ceramide may have several roles to play in eukaryotic cells, including stimulation of second messenger system, activation of mitogen-activated protein kinases [MAPKs], changes in cell shape, and

even apoptosis based on the length of the fatty acids or the mode of metabolism [63]. This toxin enhances the host cell susceptibility to other lytic agents such as *α*-hemolysin & PVL. Lymphocytes, keratinocytes and neutrophils are susceptible to beta toxin and the toxicity is enhanced by Mg^{2+}. However, these cells are less sensitive than sheep erythrocytes due to the presence of less sphingomyelin [64] as susceptibility of various cell types is based on sphingomyelin content.

Table 3. *α*-hemolysinin Drug Discovery/Therapeutic development.

Toxin/Therapeutic target	Type of inhibitor and specifications	Potential mechanism of action	Phase of development	Ref
α-hemolysin	Anti-Hla MAb MAbs 7B8 & 1A9	Antagonizes the toxin activity and block the formation of *α*-hemolysin oligomer on the target	Mice pneumonia model	[44]
	Anti-Hla MAb High affinity MAb 2A3.1	Inhibits toxin mediated cell lysis by blocking the formation of toxin heptamers on erythrocyte membranes	Independent animal models, *S.aureus* mouse dermonecrotic model and pneumonia model	[45]
	MAb MAb LTM14	Prevents the binding of toxin to the plasma membrane of susceptible host cells	Mice pneumonia, skin and bacteremia models	[47]
	Chemical compound ANBO*β*CD (*β*-cyclodextrin derivatives)	Blocks the trans membrane pores and prevents the ion leakage through the pores	Mice pneumonia model	[49]
	Chemical compound Isatin-Schiff copper (II) complexes	Prevents the formation of ion channels by obstructing the constriction region of the hemolysin channel	*In vitro* assays	[52]
	Chemical compound ADAM10 inhibitor (GI254023X)	Inhibits binding of *α*-hemolysin to its host receptor (ADAM10)	Mice model of recurrent skin and soft tissue infection	[57]
	Natural compound Oroxylin A, Oroxin A and Oroxin B	Binds to the stem region of *α*-hemolysin and prevents conformational transition of toxin from monomer to oligomer	*In vitro* assays	[51]
	Natural compound Morin hydrate	Inhibits self-assembly of the heptameric trans membrane pore of *α*-hemolysin	Mice pneumonia model	[53]
	Natural compound Curcumin	Inhibits the pore forming	Mice pneumonia model	[58]
	Natural compound Baicalin	Interrupts the formation of heptamer	*In vivo* assays	[59]

2.2.3. Mode of Action

β-toxin structure is similar to that of sphingomyelinases produced by *Listeria ivanovii*, *Leptospirainterrogans* and *Bacillus cereus* [65]. Biological activities of β-toxin including hemolysis and lymphotoxicity are due to the sphingomyelinase activity of the enzyme. Its structure indicates that it belongs to the DNase I superfamily and is it possible that β-toxin might cleave DNA [66] (**Figure 4**).

2.2.4. Pathophysiology

Epidemiological studies showed that β-toxin plays a role in the recurrence of *S. aureus* furunculosis, chronic osteomyelitis and respiratory infections in humans [67] [68]. It also contributes to biofilm formation and this activity is independent of sphingomyelinase activity [69] Animal studies revealed that β-toxin is a virulence factor for *S. aureus* induced keratitis [67] and mastitis [70]. It reportedly causes neutrophil-mediated lung injury, through both its sphingomyelinase activity and syndecan-1 [66]. Purification of β–toxin includes gel-filtration, precipitation, ion-exchange chromatography, gel electrophoresis and isoelectric focusing [5] (**Table 4**).

2.3. γ-Hemolysin

2.3.1. Discovery

Gamma hemolysin was first described by Smith and Price in 1938. It is able to lyse erythrocytes from a wide range of mammalian species [72] like human, rabbit, and sheep though rabbit RBCs were more sensitive and those of fowl were most resistant. The gene encoding γ-hemolysin is quite ubiquitous in (>99.5%) human isolates of *S. aureus* [73]. *Staphylococcus* aureus 5R is a good source of gamma hemolysin as it does not produce any other hemolysins [74].

2.3.2. Chemical Nature of γ-Hemolysin

γ-Hemolysin and Leukocidin are two component toxins located in two distinct loci within the *S. aureus* genome and a high level of sequence identity is present between these genes. The γ-hemolysin gene is located within a 4.5 kb *ScaI* fragment of *S. aureus* chromosome. It is another type of pore forming toxin, unlike α-hemolysin [75]. The *hlg* consists of two transcription units, (the first one encodes gamma hemolysin-A (Hlg A) like protein, a class S component, and the second one codes gamma hemolysin C (Hlg C) & gamma hemolysin B (Hlg B), a class of F and S components. These three proteins are translated as a single protein which is cleaved to form mature proteins which get secreted. Molecular weights of these proteins are 32,000 for Hlg A, 32,500 for Hlg C, 34,000 for Hlg B and their pI values are 9.4, 9.0, and 9.1 respectively [76].

Table 4. Drug discovery and development.

Toxin/ Therapeutic target	Type	Compound	Potential mechanism of action	Phase of development	Ref
β-hemolysin	MAb	dAb.6X his clones	Inhibits the beta-hemolysin activity	*In vitro*	[71]

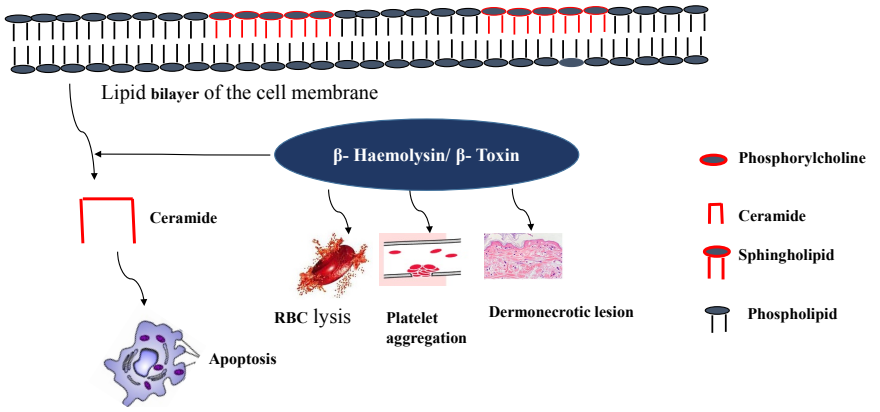

Figure 4. Interaction between *β*-toxin and cell membrane. Sphingomyelinase hydrolyzes the sphingomyelin into ceramides and phosphorylcholine.

2.3.3. Mode of Action

Like *α*-hemolysin, *γ*-Hemolysin and Panton-Valentine Leukocidin (PVL or Luk SPV and LukFPV) are also membrane-bound hetero oligomers on the surface of target cells, creating barrel-like pores which lead to cell lysis [72]. Binding studies revealed both S and F components are necessary for the biological action, S-component binds first and recruits the F-component to the surface of phagocytic cells, which is followed by oligomerization and cell lysis [77]. Structure of these bi-component toxins and their mechanism of pore formation are extensively studied. Gamma hemolysin is not identifiable on blood agar plates due to the inhibitory effect of agar on the toxin activity [73]. *Staphylococcus aureus* reportedly expresses up to five different bi-component leukocidins which include *γ*-Hemolysin (HlgAB and HlgCB), Panton Valentine Leukocidin (PVL or LukSF), LukED and Luk GH [78] (**Figure 5** and **Figure 6**).

2.3.4. Pathophysiology

Gammahemolys in is able to enhance the survival of *S. aureus* in human blood [80]. *Ex vivo* findings revealed that *S. aureus γ*-hemolysin is associated with blood stream infections including bacteremia and septic arthritis in mouse models [35] [80] and endophthalmitis in rabbits [81]. It also plays a role in the pathogenesis of toxic shock syndrome (TSS) [82]. Gamma-hemolysin has been shown to be dermonecrotic in rabbits [83] (**Table 5**).

Table 5. Drug discovery and development of therapy.

Toxin/Therapeutic target	Type	Compound	Potential mechanism of action	Phase of development	Ref
γ-hemolysin	Natural compound	Ponciretin	Inhibits the hemolytic and cytolytic activity of the toxin	Docking agent	[84]
	Natural compound	Garcinia pedunculta & 1,3,6,7-tetra hydroxyxanthone	Inhibits the hemolytic and cytolytic activity of the toxin	Docking agent	[85]

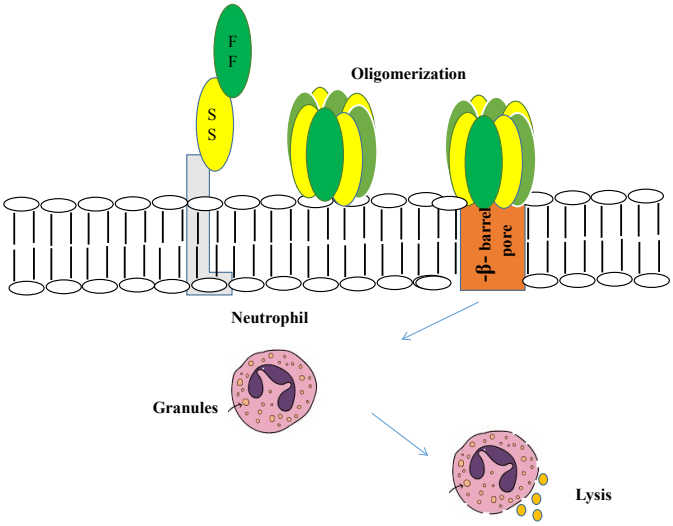

Figure 5. Interaction between γ-hemolysin and cell membrane. *S. aureus* produces the bi-component γ-toxin as two sub units (green and yellow) which bind to the receptor, oligomerize and create pores in the membrane leading to cell lysis.

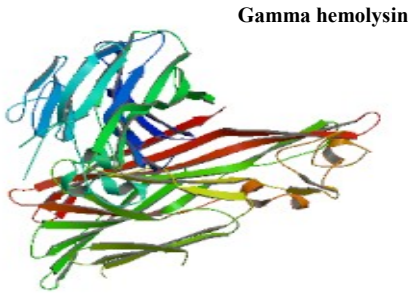

Figure 6. Structure of Gamma-hemolysin (PDB ID: 2QK7) [79].

2.4. Panton-Valentine Leukocidin (PVL)

Panton-Valentine Leukocidin is extracellular cytotoxin and it was first described by Van de Velde in 1894 and it was first purified by Woddin from *S.* aureus V8 culture supernatants [86]. Human and rabbit neutrophils are highly sensitive to PVL [87] and this gene spreads among *S. aureus* due to a combination of clonal expansion and horizontal transfer [88]. It contains two synergistic but separate protein components (32 kDa and 38kDa) named as "S" slow eluted and "F" fast eluted on the basis of their migration on carboxy methyl cellulose columns [89]. It forms pores in the membranes of leukocytes and leads to lysis. The genes of *lukF-PV* and *lukS-PV* have been recently cloned and sequenced [90]. It mainly targets the polymorphonuclear cells [91].

Panton & Valentine first revealed in 1932 that this leukotoxin was associated with skin and soft tissue infection [86]. Association between PVL positive *S. aureus* isolates and community-acquired (CA) pneumonia was reported by Lina *et al.*, through PCR assay [92], and these studies were later correlated with studies conducted in France, Sweden, Netherlands and the United Kingdom [93] [94] [95] [96]. PVL is highly prevalent among CA-MRSA strains world wide. Studies

revealed that 98% of CA-MRSA isolates were positive for the PVL gene [97] and is considered as a marker for the detection of CA MRSA isolates worldwide [92] [98]. Clinical studies propose that PVL is a crucial virulence factor in necrotizing disease [92] [99].

2.5. Delta-Hemolysin (Hld)

Williams and Harper in 1947 detected for the first time delta hemolysin activity in sheep blood agar plates wherein alpha and beta hemolysins were inhibited by respective antibodies [100]. Delta-hemolysin is a small amphipathic peptide toxin consisting of 26 amino acids, produced by almost all isolates (97%) of *S. aureus*. Molecular weight of this protein is approximately 3000 daltons [101] and it is secreted without signal peptide. A variety of activities have been attributed to delta hemolysin. Its activity is not inhibited by ethylene diamine tetra acetic acid, citrate or metal cations while it is uniquely inhibited by serum components, possibly the serum proteins or phospholipids [100]. The toxin acts as a surfactant to disrupt the cell membrane resulting in membrane damage in a variety of mammalian cells, as well as membrane-bound organelles [101]. The toxin lyses the cell by three different mechanisms: first, it binds and forms transmembrane pores, next it destabilizes the membrane and at high concentrations, it acts as a detergent that dissolves the membrane [102].

A 514-nucleotide transcript is encoded by the *hld* gene located within the RNAIII locus of accessory gene regulator (*agr*) which controls the expression of virulence factor [7]. Delta hemolysin is controlled by *agr* and maximum expression is found in post exponential phase. This hemolysin causes a rapid influx of Ca^{2+} and stimulates free radicals production in human granulocytes [101]. Among the hemolysins produced by *S. aureus,* delta hemolysin activity seems to be accentuated (enhanced) by *β*-hemolysin [103].

Delta hemolysin activates neutrophils which lead to the generation of Reactive Oxygen Intermediates (ROI) and platelet activating factor, inducing the release of granule enzymes and modulating leukotriene generation [104]. Delta hemolysin has a significant role in the development of skin disease, atopic dermatitis, due to mast cell degranulation [104]. It also induces the release of pro-inflammatory cytokines from keratinocytes [14]. It is transcribed from a promoter immediately upstream of the *agr* locus, and which is positively controlled by *agr*-QS system. Production of delta hemolysin can be used as a surrogate marker of *agr* function [105] [106]. Recent studies revealed that *agr* function can be assessed by the evaluation of delta toxin production from whole bacterial cells using whole cell matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry [107].

3. Accessory Gene Regulator (AGR) Quorum Sensing (QS) System

Staphylococcal *agr*-QS system is a well-studied central transcriptional regulator for controlling the expression of staphylococcal virulence factors including the

hemolysins, such as α, β, γ and δ-hemolysins. It is encoded by the *agr* locus which consists of two transcription units driven by P2 & P3. P2 operon encodes RNA II, consists of four proteins to activate the QS system, of which Agr C is the receptor for auto inducing peptide (AIP) and Agr A is the response regulator to activate the transcription of P2 & P3 and two membrane proteins, Agr B and Agr D which combine to produce and secrete an Auto Inducing Peptide (AIP). The *agr* P3 promoter drives the synthesis of RNA III, the major regulator of *agr* QS system [7]. Part of the RNA III encodes the *hld*, and therefore the expression of *hld* serves as a surrogate marker to assess *agr* functionality (**Figure 7**).

4. Methods for Detecting Hemolysins

4.1. Qualitative Methods

It was reported in a classical study of *S. aureus* from human clinical isolates [4] that 96% produced α-hemolysin, 11% produced β-hemolysin and 97% produced δ-hemolysin though γ-hemolysin was not reported (probably due to inhibition by agar). Hemolysins α and δ are more common among human isolates while β hemolysin is detected in addition to these two, in animal isolates of *S. aureus.* Clinical isolates are cultured on blood agar plates using Columbia blood agar powder Supplemented with rabbit erythrocytes (α-hemolysin), sheep erythrocytes (β-hemolysins) are used for the detection of α & β hemolytic activity [108]. γ-hemolysin activity is determined on rabbit blood in agarose plate instead of agar plate as agar inhibits the γ-hemolysin activity [109]. Delta hemolysin activity is detected and semi-quantitated by cross-streaking of test isolate perpendicular to *S. aureus*25923. The latter is a reference strain which produces only β-hemolysin on sheep blood agar plates. A zone of enhanced hemolysis is seen at the junctions where β & δ lysis zones overlap [110]. Original results of a representative experiment demonstrating various hemolysin activities on blood agar plates are presented from our laboratory (**Figure 8**).

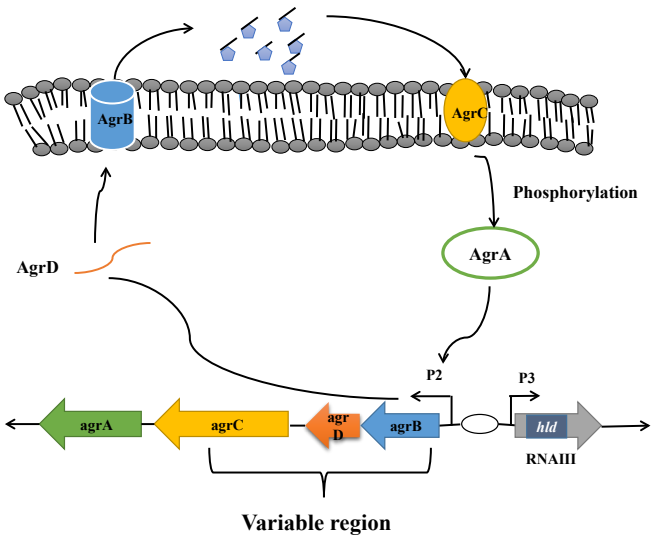

Figure 7. *Agr* QS system.

(a) (b) (c)

Figure 8. Hemolytic activities of *S. aureus* on Sheep blood agar plate: (a) *δ*-hemolysin activity on Sheep blood agar plate (b) *β*-hemolysin activity on Sheep blood agar plate (c) *α*-hemolysin activity on Rabbit blood agar plate (data from our laboratory).

4.2. Genotypic Methods for Detection of Hemolysins

Hemolysin genotyping is done using specific oligo primers to amplify target sequences by PCR from the *hla*, *hlb*, *hlg*, *hld* genes which encode the hemolysins alpha, beta, gamma and delta respectively in *S. aureus* [108].

4.3. Quantitative Methods

4.3.1. *α*-Hemolysin Assay

Functional assay is used to measure the hemoglobin released from the rabbit erythrocytes by the hemolytic activity of *α*-hemolysin [111]. Briefly, rabbit erythrocytes were centrifuged and washed three times with PBS and diluted to a concentration of 10^7 cells per ml. Rabbit erythrocytes (10^7) were added to the *α*-toxin that was serially diluted two fold in micro titer plate and incubated at 37˚C until the erythrocytes in the control lacking alpha toxin had settled. The highest dilution producing red blood cell lysis was considered the end point [49].

4.3.2. Beta-Hemolysis Assay

β-hemolysinis measured by spectrophotometer as sphingomyelinase activity and the amount of cleavage of phospholipid into sphingomyelin and phosphorylcholine is determined [67].

4.3.3. Real Time PCR

Quantitative real time reverse transcription PCR (qRTPCR) is used to measure the transcripts of *hla* & *hld* in *S. aureus* isolates and *gyr*B is used as a normalizer for *hld* [110], in case of alpha hemolysin 16S rRNA gene was used as internal or reference control [112].

5. Conclusion

Hemolysins play an important role in pathogenesis of all diseases due to *S. aureus*. They help to lyse the host cell membrane, maim or evade the immune system, release the nutrients for the pathogen's survival and progression of disease. Since *S. aureus* has become a super bug resistant to all available antibiotic, it is imperative that alternate targets are identified and drugs designed. Conventional

antibiotics target the nucleic acid and protein biosynthesis in the microorganisms and it became inevitable for the microbe to protect itself and survive which led to the development of resistance through several mechanisms. Virulence factors like hemolysins offer attractive targets for anti-virulence drug development and the chances of resistance to such anti-virulence drugs/therapeutics are very low as such drugs do not threaten the survival of the microbe As there are no new antibiotics under approval stage in any country, it is imperative that efforts are maintained to identify new targets and develop new drugs which would either limit the growth of these super bugs or at least reduce the severity of such infections through anti-virulence drugs. This review attempted to highlight the importance of hemolysins of *S. aureus* from the host as well as pathogen perspective and as potential targets for new drug development to be used as adjunct anti-virulence therapeutics or quorum quenchers to inhibit biofilm formation. Many laboratories including ours are working on the hemolysin profile of clinical isolates of *S. aureus* and to identify compounds either from natural products or through synthetic routes which would either inhibit the hemolysin synthesis or down regulate the *agr* system to effect quorum quenching. Though there are reports of several promising candidate drugs with high potential as anti-virulence drugs or as inhibitors of biofilm, none of them have been approved for clinical use yet.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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