CHAPTER-2

REVIEW OF LITERATURE

2.1. History:

Staphylococcus aureus is an aerobic or facultative anaerobic, coagulase positive organism which colonises the skin, nasal passage and axillae of humans. It occurs in grape like clusters when viewed through the microscope (Murray *et al.,* 2003).

Staphylococcus was first observed in human pyogenic lesions by Von Reckling Hausen in 1871. Pasteur in the year 1880 obtained liquid cultures of the cocci from pus and he produced abscess by inoculating them into rabbits. Scottish surgeon Alexander Ogston recognised conclusively the causative role of the coccus in abscesses and other supralative lesions. The author also named it *Staphylococcus* (Staphyle, in Greek means "bunch of grapes", Kokkos, means "berry") due to its typical occurrence of the cocci in grape like clusters in pus. Ogston had noticed that non-virulent staphylococci were also present on skin surfaces. Later, in 1884, Rosenbach named it *Staphylococcus aureus* (Murray *et al.,* 2003).

2.2. Systematic classification:

The first edition of Bergey's Manual of Systematic Bacteriology listed only six species of the genus *Staphylococcus*. The prokaryotic domain bacteria are ordered into 30 phyla (lines of development) and further into classes, orders, families and finally genera. *Staphylococcus aureus* belongs to the phylum Firmicutes, class Bacilli, order Bacillales with the family name Staphylococcaceae and genus *Staphylococcus*. *Staphylococcus aureus* is a species within the genus. Two subspecies have been described, *S. aureus* subsp. *aureus* and *S. aureus* subsp. anaerobius. This thesis deals solely with subsp. *aureus* and from here on, '*S. aureus*' refers to *S. aureus* subsp. *aureus*. Today, almost forty species (depending on the source) are assigned to the genus by well-defined genotypic and phenotypic criteria (De Vos *et al.,* 2009).

2.3. Microbiology:

The species *S. aureus* is a group of bacteria that test positive for Gram staining and appear, under the microscope, blue to purple in colour from the absorption of the stain by the thick peptidoglycan layer component of the bacterial cell wall (Beveridge, 2000). *S. aureus* readily grows on ordinary media like nutrient agar at a temperature of 37 °C and pH 7.4 to 7.6 in which it produces circular, convex smooth shiny opaque colonies, On Nutrient agar slope it produce oil paint appearance. On MacConkey agar medium, they produce small pink colonies due to its lactose fermentation. Typical *S. aureus* has a large, round, creamy smooth colonies with golden yellow colour on blood agar.

Most strains have beta or alpha hemolysis when growing on blood agar plates (Kloos and Schleifer 1975). *S. aureus* can survive for several hours on dry environmental surfaces (Neely and Maley 2000), and grow at a temperature range of 7 to 48°C. It can tolerate NaCl concentrations as high as 15 percent (Le Loir *et al.,* 2003).

Small colony variants (SCVs) of *S. aureus* produce colonies that are pinpoint in size, non hemolytic and non-pigmented. In liquid medium, uniform turbidity is produced. Selective media used for isolating *S. aureus* contain 8-10% NaCl like salt-milk agar, ludlam's medium containing lithium chloride and tellurite (Bannerman, 2003).

SCVs of *S. aureus* have slow growth rates, persist inctracellularly, and are less susceptible to antibiotics. They are associated with persistent or relapsing osteomyelitis and device-related infections (Proctor *et al.,* 2006 and von Eiff *et al.*, 2006).

2.4. Pathogenesis:

S. aureus is associated with a variety of systemic infections such as dermatitis, septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis and post-surgical toxic shock syndrome with substantial rates of morbidity and mortality (Shittu and Lin, 2006). Hospitalized patients are particularly susceptible to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (Lindsay and Holden, 2004).

It is a common cause of skin and subcutaneous infections, including folliculitis, furunculosis, cellulitis, mastitis, and impetigo. Skin infections left untreated can, however, develop into more serious infections such as bloodstream infection and septic shock (Lindenmayer *et al.,* 1998; Lina *et al.,*1999;).

In bovines, it is an important pathogen causing mastitis (Quinn *et al.,* 2000). *S. aureus* cause chronic and deep infection in mammary glands which is very difficult to treat (Hassan *et al.,* 2010). Bovine mastitis is responsible for reduced milk production, decline in milk quality, and increased labour cost (Beck *et al.,* 1992). Clinical and subclinical mastitis due to *S. aureus* are recognised most important disease conditions which cause huge economic losses to the dairy industry (Taverna *et al.,* 2007).

2.5. Epidemiology:

S. aureus grows harmlessly on the moist skin of the nostrils in many healthy persons. This condition is referred to as colonization. General colonisation of the skin, such as the arms, toes and forehead also occurs, but this is usually considered as transient carriage (Crossley and Archer, 1997).

Staphylococci organisms are wide spread in nature although they are mainly found as normal flora on the skin, skin glands and mucous membrane of mammals and birds. Carriage of *S. aureus* is estimated to occur in 15 – 100 % of individuals depending on the age, demography and health of the population being sampled, and is categorised as either transient or persistent carriage. Persistent carriage exists in 20 – 35 % of a healthy population, with the remaining 65- 80% of the population experiencing transient carriage, which can last from as little as a few days, up to a few weeks (VandenBergh *et al.,*1999).

The anterior nares (nostrils) are the site most frequently associated with *S. aureus* carriage (Coates *et al.,* 2009), with three distinct patterns in the population: persistent carriers (20%), intermittent carriers (60%), and non-carriers (20%) (Foster, 2004). However, it can also be isolated from the perineum, the pharynx and the axilla (Dancer and Noble, 1991).

The presence of enterotoxigenic strains of *S. aureus* in various food products is regarded as a public health hazard, because of the ability of these strains to produce intoxication or food poisoning. *S. aureus* are major species of primates, although specific serovars or biotypes can be found occasionally living on different domestic animals or birds (Murray *et al.,* 2003).

Colonisation distribution can vary considerably if the population being sampled is somehow compromised. HIV/AIDS patients have been shown to have been reported to have high persistent nasal carriage (Padoveze *et al.,* 2008), and eczema sufferers tend to have increased persistent generalised skin colonisation (Crossley and Archer, 1997). Nasal colonisation has been found to have a strong link with infection (Kluytmans *et al.,* 1997; Kalmeijer *et al.,* 2000). Patients who tested positive for nasal carriage were three times more likely to develop infection than non-colonised patients (Wertheim *et al.,* 2004). This poses a risk for patients admitted for surgical procedures which may provide a route of entry for the bacteria.

Although the majority of these reports focused primarily on MRSA, the potential for the occurrence and transmission of a complex assembly of methicillin-resistant *Staphylococci* has not been evaluated. This is important, since *Staphylococci* that were historically regarded as non-pathogenic (Thylefors *et al.,* 1998) are now known to pose a high risk of infection and has emerged as significant clinical and community pathogens (Vengust *et al.,* 2006).

Bradshaw (2003) provided evidence that MRSA could be transmitted from dogs to humans, while Sing *et al.,* (2008) reported that isolation of MRSA from cat and it's infected owner, therefore implicating the cat as a potential source of the pathogen. Recent evidence collected from SCCmec typing showed that MRSA isolates retrieved from companion animals were identical to epidemic strains occurring in hospitals (Leonard and Markey, 2008).

Transmission of *S. aureus* in the health care setting occurs through contact with contaminated surfaces in the environment (Boyce, 2007). *S. aureus* and MRSA have been shown to persist for weeks on items in the hospital such as patient care equipment, uniforms, computer key boards, cellular phones and identification badges (Kramer *et al.,* 2006).

In a study, Fluit *et al.,* (2001) reviewed in several industrialized nations, including parts of Europe, the USA and Japan, 40–60% of all hospital *S. aureus* are now resistant to methicillin (MRSA).

Recently Ko *et al.,* (2005) reported the distribution of a single genotype of *S. aureus* (collected from hospitals in 12 countries) among different Asian countries, including China, India, Indonesia, Philippines, Saudi Arabia, Singapore, Sri Lanka, Taiwan, Thailand, and Vietnam. This suggests low genetic diversity but a high persistence of the pathogenic strains in different areas.

Therefore, the possibility of spread of such strains from hospitals to the community cannot be ignored.

Various food products are frequently contaminated by *S. aureus* and incriminated for *S. aureus* food poisoning including meat products, eggs, dairy products, vegetables and other processed food such as sandwich fillings or chocolate éclairs (Normanno *et al.,* 2005).

MRSA has been isolated from meat or dairy products in several countries including Netherlands, Italy, Australia, Japan and United States (Kitai *et al.,* 2005; Normanno *et al.,* 2005; Pereira *et al.,* 2009; Pu *et al.,* 2009). Food products derived from the animals may be contaminated with *S. aureus* or MRSA during slaughtering and processing (Vanderhaeghen *et al.,* 2010).

Many skin infections are reported to be caused by CA- MRSA strains. It has been suggested that prevalence of CA- MRSA and morbidity and mortality associated with these strains will be constantly witnessed all over the world. Due to accessibility of inadequate number of population based studies community-acquired MRSA infections exists a world-wide.

It has now become important to differentiate between CA-MRSA and HA-MRSA in order to provide ideal treatment and control measures, as well as to consistently monitor the epidemiological MRSA situation worldwide. Now-a-days it has been evaluated that an MRSA infection manifesting in a community setting may be caused by an MRSA strain acquired by some earlier hospital stay, but become falsely identified as CA-MRSA based on epidemiological information. Interestingly, most bacteremias diagnosed at hospital admission, and therefore classified as CA-MRSA infections, have been caused by hospital associated (HA-MRSA) from a previous healthcare contact (Miller, *et al.,* 2008). On the other hand, MRSA infections developing during hospital stay may be caused by MRSA acquired in the community. Epidemiological

classification was more useful in the past than nowadays. Although HA-MRSA is still uncommonly transmitted outside hospitals, CA-MRSA clones have begun to spread in hospitals (Otter & French, 2012).

2.6. Geographic distribution of Methicillin-Resistant *Staphylococcus aureus* (MRSA):

MRSA can be found worldwide, but its prevalence varies (Weese *et al.,* 2006; Cuny *et al.,* 2006). Human-adapted, hospital associated strains of these organisms are rare among people in the Netherlands and Scandinavian countries, where extensive control programs have been conducted for years (Leonard and Markey, 2008; Kluytmans, 2010; Otter and French, 2010). Community-associated strains can occur even where hospital-associated strains have been controlled (Otter and French, 2010).

2.7. Evolution of MRSA:

In the past *S. aureus* infections were treated with penicillin, however within two years of the introduction of penicillin to medical use, penicillin-resistant strains were discovered. By 1960, about 80% of all *S. aureus* strains were found to be resistant to penicillin (Deurenberg *et al.,* 2007).

Methicillin was introduced in 1959 to treat infections caused by penicillin resistant *S. aureus* (Enright *et al.,* 2002). But later, the organism have become resistant to various drugs including methicillin and its related antibiotics. These resistant bacteria are called Methicillin resistant *S. aureus* or MRSA (CDC). Since the emergence of methicillin-resistant *S. aureus,* the glycopeptide vancomycin has been the only effective treatment for *Staphylococcal* infections.

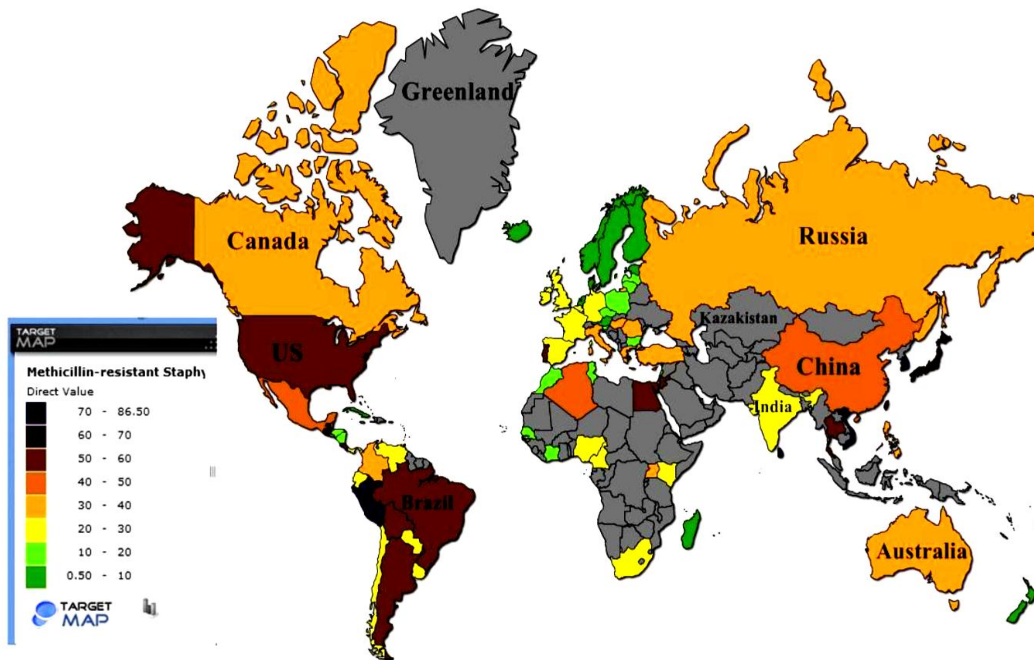

Figure 2.1 Global prevalence of MRSA (Modified from http://www.targetmap.com)

Resistance to β-lactam antibiotics arise in two ways in *S. aureus*. The first mechanism is by the production of β-lactamase enzymes, products of the *blaZ* gene, which hydrolyse the β-lactam ring, a structural component of the antibiotic, rendering it ineffective (Thumanu *et al.,* 2006). Alternatively, resistance can be via the acquisition of the *mecA* gene responsible for the production of an additional penicillin binding protein PBP2' or PBP2a, which has a different conformation to the normal PBP (Hartman and Tomasz, 1984). These changes prevent methicillin and other β-lactams from binding to the PBP, resulting in continued peptidoglycan synthesis and redundancy of the antibiotic family (Lim and Strynadka, 2002).

Methicillin resistance in *S. aureus* (MRSA) was first reported from UK in 1961 (Barber, 1961). It is now found world-wide and with increasing incidence (World Health Organisation, Monitoring of Antimicrobial Resistance, 2003). Between 2004 and 2006, seven epidemic MRSA strain types were defined to be community-associated (Kanerva, *et al.,* 2009).

In May 1996, Japan documented for the first time clinical infections due to *S. aureus* that acquired vancomycin resistance (Hiramatsu *et al.,* 1997). The first case of VISA was reported in Japan in 1996, but the first case of *S. aureus* truly resistant to glycopeptide antibiotic was reported in 2002. As of 2005, 3 cases of VRSA had been reported in the US (Ito *et al.,* 2001).

MRSA strains have been identified in different food products, including meats and raw milk (Nomanno *et al.,* 2007; de Boer *et al.,* 2009). Transmission of foodborne pathogens from animals, including dairy cattle, has been investigated in a number of different countries (Chao *et al.,* 2007; Normanno *et al.,* 2007)

Hussian *et al.,* (2000), reported overall prevalence of community acquired MRSA was 208 per 1, 00,000 admissions. Twenty-three hospitalized children had an MRSA, ten were community-acquired, equally distributed between children with predisposing risk factors and those without.

Price *et al.,* (2000), investigated Methicillin resistant *S. aureus* nasal colonization in patients with elective cardiovascular surgery, renal patients admitted for arteriovenous graft surgery, and patients transferred from other hospitals. Renal failure patients were significantly more likely to represent a potential source of MRSA to their institution.

In an open study Shetty and Wilson, (2000) described patients with serious systemic infections with methicillin resistant *S. aureus* (MRSA) treated with glycopeptides. Of 11 patients with MRSA infection, four were cured, six were failed with treatment and one was intermediate.

Wong *et al.,* (2000) investigated the geographical distribution of 65 clinical isolates of methicillin resistant *S. aureus* (MRSA) recovered from 7 hospitals in Thailand and identified presence of *mec A* gene in MRSA.

Miall *et al.,* (2001), studied Prevalence of MRSA raised from 0 in 1992- 94 to 7 in 1998 from a clinic population of 30,010 children having positive sputum cough swab cultures for MRSA.

Hendriksen *et al.*, (2008) in a survey from 2002 to 2004, found that *S. aureus* isolates from more than 9 out of 13 European countries were susceptible to most of the antibiotics tested. However, they mentioned that over 10% of *S. aureus* isolates from 10 countries were resistant to penicillin.

The CDC has proposed 5 factors (or 5 Cs) associated with CA-MRSA transmission, i.e., 1) Crowding, 2) frequent skin-to-skin Contact, 3) Compromised skin integrity, 4) Contaminated items and surfaces, and 5) lack of Cleanliness. Wrestlers, football players, prison inmates, soldiers, children in day-care centers, etc. have been identified as high risk groups for CA-MRSA (Liu, *et al.,* 2008).

Chambers and DeLeo, (2009) compared the multidrug resistance (MDR) pattern between HA-MRSA, and community associated MRSA (CA-MRSA) and found CA-MRSA strains have less often been MDR, i.e. resistant to more than 3 different antimicrobial groups.

In a study by Hoerlle and Brandelli, (2009) showed higher glycopeptides activity against the isolates of *S. aureus* (100% of susceptibility for vancomycin and teicoplanin). The percentage of samples showing resistance to at least one drug was 96%, 97% and 100% for the years 2001, 2003 and 2004, respectively. Except for ampicillin and penicillin, antimicrobial resistance decreased from 2001 to 2004.

Abera *et al.*, (2010), in their study reported that *S. aureus* was commonly resistant to penicillin (94.4%) and amoxicillin (36.1%), while susceptible to other antimicrobial agents such as chloramphenicol and gentamicin using the disk diffusion method.

DeLeo *et al.,* (2010) reported that CA-MRSA is transmitted by direct contact with infected or colonized individuals, or through a MRSA-contaminated environment. CA-MRSA has been reported to be acquired through activities in which direct body contact is common.

Shi *et al.,* (2010), found that, in general, *S. aureus* resistance in China was high against nine groups of antimicrobial agents tested in their study using the disc diffusion method. They found that 232 out of 236 (98.3%) isolates were resistant at least to one of the antimicrobial drugs, and the isolates were most commonly resistant to penicillin (87.6%).

Kumar *et al.,* (2011) reported a high prevalence of MRSA (13.1%) from milk samples of 195 infected udders. The isolates were also highly resistant to antibiotics, i.e. 36.4% were resistant to streptomycin, 33.6% to oxytetracycline, 29.9% to gentamicin and 26.2% each to chloramphenicol, pristinomycin and ciprofloxacin.

According to a recent review by Stefani, *et al.,* (2012) it was found that the prevalence of hospital associated MRSA (HA-MRSA) around the world follows the highest rates (> 50%) from North and South America, Asia and Malta. Intermediate rates of 25-50% were reported from China, Australia, Africa, Portugal, Greece, Italy, Hungary, Spain, Ireland, Romania, and the United Kingdom. The northern European countries stand out with their low rates of < 5%, while the prevalence in the rest of Europe is currently 5-25%.

New antimicrobial agents, such as linezolid has been recently introduced to the treatment of MRSA infections. Linezolid is a bacteriostatic agent effective against Gram-positive cocci including MRSA (Ager and Gould, 2012). Interestingly, *S. aureus* strains resistant to linezolid have already emerged (Morales *et al.,* 2010).

2.8. Genome organization:

From the sequenced *S. aureus* strains, the range of genomes size is around 2.8 to 2.9 Mbp (Holden *et al.,*2004; Lindsay and Holden, 2006), which include 75% essential genes for cell surviving and other accessory genome, bacteriaphages and pathogenicity island that contains various virulence genes (Kuroda *et al.,* 2001; Holden *et al.,* 2004; Lindsay and Holden, 2006). *Staphylococci* have a low percentage (30% – 39%) of guanine plus cytosine (G+C) in their genetic make-up when compared to other gram positive cocci (Prescott *et al,.* 1996).

SCC*mec* is the genetic material of 21-67 kb in size which is present in the chromosome of MRSA strains on the unique site known as *attBscc.* This site is located near origin of replication. *attBscc* occurs as open reading frame of unknown function which is identified as *orfX.* SCC*mec* are variable genetic materials which have certain conserved features. Among the conserved elements, SCC*mec* contains the *mec* operon which is composed of *mecA* and its regulatory gene as well as the cassette chromosome recombinase complex *ccr* (Holden *et al.,* 2004). The variable regions of SCC*mec* are known as J-regions which contain integrated genetic elements like plasmids (pT181, pUB110 and p1258), transposons (Tn554) and insertion sequences (IS431, IS1272 and IS256). Based on the combination of different *mec* and *ccr* complexes, 8 types of SCC*mec* elements have been defined on the basis of combination of different *mec* and *ccr* complexes till date (Zhang *et al.,* 2009).

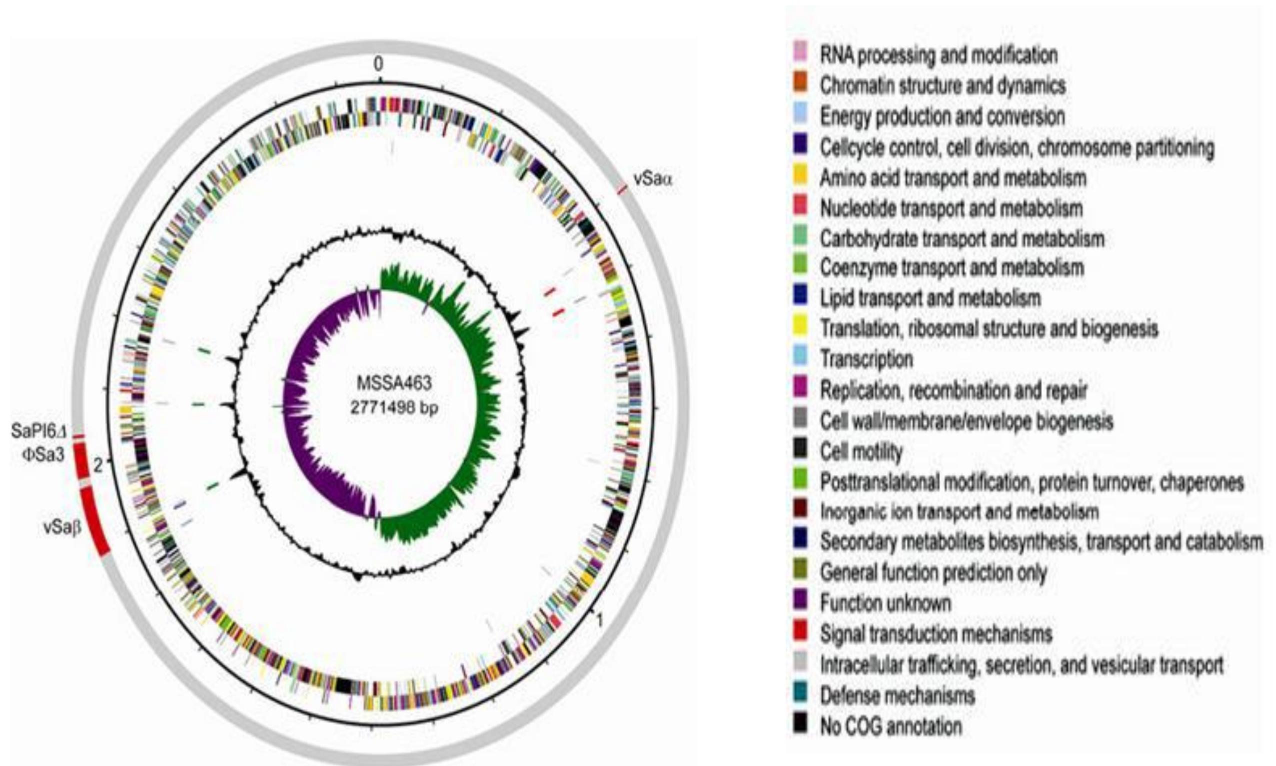

Figure 2.2. Schematic circular diagram of the SCC-like MSSA463 chromosome. Where appropriate, categories are shown as concentric circles representing coding strands. From the outside to inside: the colored segments on the gray outer ring represent genomic islands and horizontally acquired DNA. Inside the gray outer circle, the second circle represents the nucleotide position in Mb. The third and fourth circles show open reading frames (ORFs) on the plus and minus strands, respectively. Different colors represent different Clusters of Orthologous Groups (COG) functions. The color coding for coding sequences (CDS) is listed to the right of the circular diagram. The fifth circle indicates the location of predicted tRNAs. The sixth and seventh circles represent rRNA predicted on the plus and minus strands, respectively. The eighth and ninth circles show G+C content and G+C skew, respectively (> 0%, green; <0%, purple), (De Zhi *et al.,* 2013).

2.9. Virulence factors of *S. aureus*:

S. aureus is equipped with a wide arsenal of virulence mechanisms (Table 1). These enable

abscess formation, evasion of host immune responses at many levels, and induction of the sepsis syndrome (Raygada and Levine, 2009). Nearly all strains secrete a variety of proteins to the surrounding media that convert host tissues to nutrients for the invading bacterium (Dinges *et al.,* 2000).

Table 2.1. *S. aureus* virulence mechanisms (Modified from Raygada and Levine, 2009)

Virulence Factors	Description	Role	Clinical Manifestations
clumping factors	surface	attachment to host tissues	Endocarditis,
fibrinogen binding protein	proteins		foreign body infections
collagen binding protein	MSCRAMM[a]		septic arthritis
proteases			
lipases			
nucleases	invasins	penetration into host tissues	invasion and destruction
hyaluronidases			
elastase			
haemolysin	cytotoxins	lysis of red blood cells	metastatic infections
PVL[b]		destruction of leucocytes	necrotizing pneumonia
Capsular polysaccharides		evasion of opsonization	persistent infections
protein A		biofilm production	abcscess formation
TSST[c]	exotoxins	releases cytokines	toxic shock syndrome
enterotoxins		gastrointestinal toxins	food poisoning
exfoliative toxins		cleaves epidermal structures	scalded skin syndrome
penicillinase	resistance	cleaves penicillin	penicillin resistance
PBP2a[d]	determinants	altered antibiotic binding site	methicillin resistance
VanA [e]		altered antibiotic binding site	vancomycin resistance

[a]MSCRAMM, microbial surface components recognizing adhesive matrix molecules

[b]PVL, Panton-Valentine leucocidin

[c]TSST, toxic shock syndrome toxin

[d]PBP2a, an altered penicillin binding protein encoded by the mecA gene

[e]VanA , a gene encoding vancomycin resistance

2.9.1. Biofilm as a virulence factor:

Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime), which facilitates the adherence of microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy (O'Gara and Humphreys, 2001). *Staphylococci* are most often associated with chronic infections of implanted medical devices. The pathogenesis of device associated infections with *Staphylococci* is mainly characterized by the pathogen's ability to colonize the surfaces of the implanted medical device by the formation of the biofilm which is composed of polysaccharides, proteins, extracellular DNA, and host factors. Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA), which mediates cell to cell adhesion and is the gene product of *icaADBC* (Ammendolia *et al.,* 1999). Recent studies indicated that among clinical isolates of *S. aureus*, only between 45% and 70% (depending on the type of infection) strains were able to form biofilm (Grinholc *et al.,* 2007).

There are 2 major steps in the formation of biofilm (Cramton *et al.,* 1999). Firstly there is a requirement for attachment of the bacterial cells to a surface, so called early adhesion. The second action is growth-dependent accumulation of bacteria in multilayered cell clusters which requires intercellular adhesion (Heilmann *et al.,* 1996; Cucarella *et al.,* 2001).

2.9.1.1. The *ica* Gene Locus in biofilm formation:

The *ica* operon *(icaADBC)* consists of 4 open reading frames, with an assumed regulatory gene *(icaR)* upstream and in the opposite orientation (Cramton *et al.,* 1999; Cramton *et al.,* 2001). *icaA* and *icaD* work synchronously to produce sugar oligomers from UDP-N-acetylglucosamine in *in-vitro* assays (Cramton *et al.,* 2001).

icaA encodes a trans-membrane protein which has N-acetylglucosaminyltransferase activity, but is only fully active in the presence of the product of *icaD*. It is believed that *icaC* also aids mobilisation of the polysaccharide produced by *icaADC* to the cell surface (Gerke *et al.,* 1998). *IcaB* is responsible for deacetylation of the polysaccharide increasing its positive charge and is thought to enable attachment of the polysaccharide to the cell surface (Vuong *et al.,* 2004; Cerca *et al.,* 2007) (Figure 2.2).

Additionally, Cramton *et al.,* (1999) demonstrated the presence of the *icaA*DBC operon in other staphylococcal species, suggesting that the cell–cell adhesion mediating the *icaA*DBC operon is highly conserved within this genus. However, while *icaADBC* gene locus is currently the best understood mechanism of biofilm formation, more recent studies have shown that biofilm formation is possible independent of the *ica* genes (Fitzpatrick, Humphreys & O'Gara, 2005; Toledo-Arana *et al.,* 2005).

A novel surface protein has been isolated which is directly associated with biofilm formation on abiotic surfaces *in vitro* in the absence of host plasma constituents called biofilm associated protein (Bap). This novel protein has marked similarities to surface proteins previously discovered on gram-negative *(Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and gram-positive *(Enterococcus faecalis)* micro-organisms. All bap-positive *S. aureus* strains tested in one study were highly adherent and strong biofilm producers due to this abundance of surface proteins (Cucarella *et al.,* 2001).

2.9.1.2. *ica* independent biofilm formation

An alternative, *icaADBC*-independent, mechanism of biofilm formation by *S. aureus* isolates causing mastitis has been described by Penadés and Lasa. In their research on the *S. aureus* bovine mastitis isolate V329, they proved that the biofilm-associated protein (Bap) instead of PIA was indispensable for the primary attachment and cells' accumulation (Cucarella *et al.,* 2001; Lasa and Penadés, 2006).

The biofilm structure may depend on the nature of the molecules involved in biofilm formation. BLAST searches (Altschul *et al.,* 1997) for sequence homologues to Bap showed the existence of a novel family of proteins, previously named BAP (Biofilm Associated Proteins; Cucarella *et al.,* 2004), which are important for biofilm formation in both Gram-positive and Gram-negative bacteria.

The presence of Bap significantly increased the ability of organisms to colonize and persist in the bovine mammary gland *in vivo*. At the same time, Bap-positive isolates were less susceptible to antibiotic treatments when forming biofilms in vitro (Cucarella *et al.,* 2004). Analysis of the structural *bap* gene revealed the existence of alternative forms of the Bap protein, which contain a different number of repeats, in *S. aureus* isolates obtained under field conditions throughout the animal's life. The presence of anti-Bap antibodies in serum samples taken from animals with confirmed *S. aureus* infections indicated the production of Bap during infection (Cucarella *et al.,* 2004).

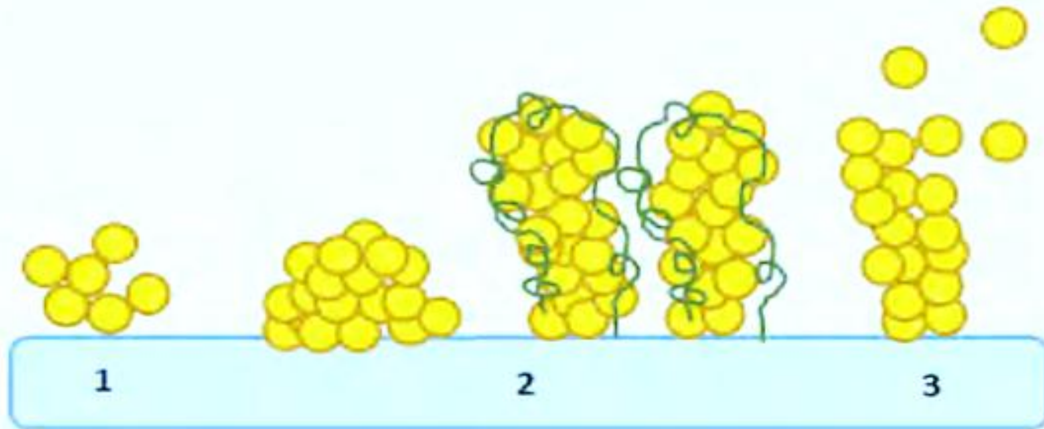

Figure 2.3. Three main stages of *S. aureus* biofilm development: 1) Attachment :to antibiotic devices mediated between microbial surface components recognizing MSCRAMMS and host proteins or non-specific interaction with abiotic polymers. 2) Maturation; and development of biofilms 3D structure. Extracellular interactions between *icaA*DBC encoded PIA/PNAG or via bacterial cell surface proteins. 3) Detachment; of single or large clumps of cells from the main biofilm body important for bacterial dissemination to other sites of infection. (Otto, 2009).

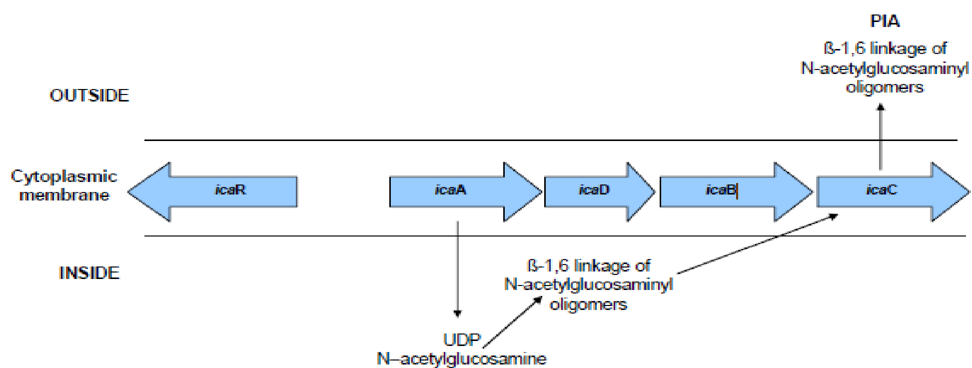

Figure 2.4. Schematic diagram of Organisation of *ica* locus in *S. aureus:* Oligomers derived from UDP Nacetylglucosamine are synthesised by *icaA*, which are then modified with longer oligomers produced by *icaD*. Deacetylation of the poly-N-acetylglucosamine molecule is carried out by *icaB* and *icaC* is finally responsible for translocation of the completed PIA to the cell surface. Adapted from Gerke *et al.*, (1998) and Gotz, (2002).

Vautor *et al.,* (2008) has reported that the bap protein is a member of proteins playing a role in biofilm formation in many bacteria and they share common structural features as they have a high molecular weight and contain a core domain of tandem repeats.

Cucarella *et al.,* (2011) studied the presence *bap* was in a small fraction of bovine mastitis isolates (5% of the 350 *S. aureus* isolates tested), but it was absent from the 75 clinical human *S. aureus* isolates analyzed. All staphylococcal isolates harboring *bap* were highly adherent and strong biofilm producers.

A work by Cramton *et al.,* (2001) and Dobinsky *et al.,* (2003) has shown a link between PIA expression in *S. epidermidis* and glucose concentration in the growth medium Maximum transcription of *ica* was detectable in the stationary phase of growth in the absence of glucose despite a PIA- and biofilm- negative phenotype. The resultant abundance of *ica* mRNA was shown to be functionally active, as induction of stationary phase cells with glucose led to immediate PIA synthesis. However, induction of biofilm formation could be completely inhibited by the addition of chloramphenicol, used to inhibit protein synthesis from the abundant mRNA upon the addition of glucose. When this was given at a later stage of biofilm accumulation, it also inhibited further development of preformed biofilm. This suggests that continuous translation of a further, *icaADBC* independent factor is required for the expression of a biofilm positive phenotype.

Vasudevan *et al.,* (2003) conducted a study to evaluate *in vitro* slime production, biofilm formation and presence of genes associated with biofilm production *i.e. ica* A and *ica* D in *S. aureus* isolated from bovine mastitic samples. They used CRA method and 32 out of 35 tested isolates produced slime while only 24 of the microbes were biofilm producer *in vitro*. However, all of the 35 sequesters contained the *ica* locus *i.e. ica* A and *ica* D genes. The experiment showed the *ica* genes among *S. aureus* mastitis isolates were highly prevalent and their presence was not

always accompanied with *in vitro* formation of slime or biofilm. They suggested that phenotypic and genotypic tests may be used in combination for determination of biofilm formation in *S. aureus.*

In a study Cucarella *et al.,* (2004) reviewed that Bap positive strains were able to form a biofilm in the absence of *ica*, suggesting that bovine isolates are also capable of *ica*-independent biofilm formation. Their findings suggest that biofilm formation in *S. aureus* is not due to the expression of a single factor, and that in different conditions, different factors may be important (Toledo-Arana *et al.,* 2005).

Fitzpatrick *et al.,* (2005) suggested environmental activation of *icaADBC* did not always correlate with increased biofilm production. Moreover, glucose-mediated biofilm development in these isolates was *icaADBC* independent. Therefore, apparently, an environmentally regulated, ica-independent mechanism(s) of biofilm development may exists in *S. aureus* clinical isolates.

Mathur *et al.,* (2006) compared the three methods of biofilm detection. To detect the biofilm trait, 152 clinical isolates of *Staphalococci* were curtained by tissue culture plate (TCP), tube method (TM) and Congo red agar (CRA) method. Of the 152 staphylococcal isolates, 57.8% (88) exhibited biofilm production when TCP method was used and strains were further categorized as high (14.47%) and intermediate (39.4%) biofilm producer organisms while 46.0% isolates were weak or non-biofilm producers. While using TM, it was difficult to discriminate between weak biofilm producers from biofilm negative isolates. The study showed that CRA method does not show a relationship with either of the other two methods for detecting biofilm formation. The results showed that the TCP method was the most profound, precise and reproducible method to detect the biofilm production by *Staphylococci.*

S. aureus surface proteins also play a role in biofilm formation due in part, to their binding activity of host matrix proteins, as described earlier. Latasa *et al.,* (2006) identified presence of

biofilm associated protein Bap, in bovine strains. However the only direct link with biofilm formation in *S. aureus, in vitro* has been the biofilm associated protein.

A study by Croes *et al.,* (2009) revealed that the MLST CC8 associated genetic background was a predisposing factor for strong biofilm formation *in vitro*, under all tested glucose concentrations. The adherence to polystyrene surfaces under physiologic glucose concentration (0.1%) was dependent on the clonal lineage. Strains associated with MLST CC8 were markedly more often classified as strong biofilm former at glucose concentrations of 0%, 0.1% and 0.25%.

Gad *et al.,* (2009) reported all biofilm producing *Staphylococci* were positive for *icaA* and *icaD* genes, among 53 Staphylococcal strains. Out of 18 *S. aureus* strains, 15 (83.3%) were biofilm producers and out of 35 *S. epidermidis* strains, 31 (88.6%) were biofilm producers. Staphylococcal strains were further classified as high (56.6%), moderate (30.2%) and non-biofilm producers (13.2%). All biofilm negative strains were negative for both genes.

Haddadin *et al.,* (2009) described the ability of *S. aureus* to produce biofilm in microtitre plates in the presence of sub-MIC antibiotics. Cefalexin induced biofilm formation at a wide range of sub-MICs. They also concluded that the antibiotics at sub-MIC levels interfere with bacterial biofilm virulence expression depending on the type and concentration of antibiotic used.

Studies by Eftekhar and Dadaei, (2011) showed that there was no agreement between the *icaAB* gene carriage and biofilm phenotype by either of the MtP and CRA phenotypic methods. 53.3% of the isolates had the potential to form biofilm by colony morphology of which, 75% carried the *ica* operon. Weak biofilm production was observed in the MtP assay by 57.8%, of which 53.8% harbored the *ica* operon. However, about 70% of biofilm non-producers also carried the *ica* operon. However, 91% of biofilm formers on CRA also produced biofilm in the MtP assay.

Esteben *et al.,* (2010) in a study reported 100% (50% *S. aureus* and 50% CoNS) strains were biofilm producers. Stepanovic test detected biofilm formation in 85% of the strains, microtiter

plate assay in 65%, and CLSM in 39%. The *ica* operon was detected in 73% of all strains. 7 *ica*-negative strains were biofilm-positive by phenotypic methods.

Lei *et al.,* (2011) reported that deletion of the *rsp* gene resulted in an increase in biofilm formation in strain MW2, suggesting that Rsp is a repressor of biofilm formation. Using SDS-PAGE, they found that Rsp profoundly affected cell surface and secreted proteins. The *rsp* gene was transcribed monocistronically, and the transcripts were most abundant at the exponential growth phase. Microarray analyses revealed that Rsp represses 75 genes, including 9 genes encoding cell wall-anchored proteins, and activates 22 genes, including 5 genes encoding secreted proteases. Among these genes, *fnbA*, *fnbB*, *sasG*, and *spa* (which encode cell wall-anchored proteins) and *splABCD* (which encode secreted proteases) have been implicated in biofilm formation.

Pozzi *et al.,* (2012), suggested that the biofilm production in methicillin-susceptible *S. aureus* (MSSA) strains is typically dependent on PIA/PNAG whereas methicillin-resistant isolates express an Atl/FnBP-mediated biofilm phenotype suggesting a relationship between susceptibility to b-lactam antibiotics and biofilm.

Kaplan *et al.,* (2012) confirmed the subminimal inhibitory concentrations ofβ-lactam antibiotics significantly induce autolysin-dependent extracellular DNA release and biofilm formation in some strains of *S. aureus*. They also reported Methicillin, ampicillin, amoxicillin, and cloxacillin induced biofilm formation in some strains. The amount of biofilm induction was as high as 10-fold and was inversely proportional to the amount of biofilm produced by the strain in the absence of antibiotics.

Goyal *et al.,* (2014) in a study reported the presence of *icaA* gene in 51.15% of the isolates and bap gene was present in 8.46% isolates. None of the isolates were positive for *icaD* gene.

Human isolates (65%) had higher occurrence of *icaA* gene in comparison to animal isolates (49.09%). Dog wound isolates had higher occurrence of *bap* gene.

2.9.2. Exotoxins as a virulent factor:

One of the important characteristics of *S. aureus* is its capability to secrete toxins that disrupt membranes of host cells. *S. aureus* produces an array of membrane damaging cytotoxins which include α-toxin, β- toxin and γ- toxin. α-toxin also called α-haemolysin is a protein inactivated at 70°C but reactivated paradoxically at 100°C because at 60-70°C the toxin combines with a heat labile inhibitor which is denatured at 100°C. It is leucocidal, cytotoxic, dermonecrotic, neurotoxic and lethal only on rabbit erythrocytes. β-haemolysin is a sphingomyelinase, haemolytic for sheep cells. γ-hemosylin is a bicomponent protein necessary for haemolytic activity (Bohach, 2000; Humphreys, 2002; Foster, 2004).

Humphrey *et al.,* (1989) found that MRSA strains isolated from septicaemic patients from Dublin hospitals, showed α, β and γ haemolysin production in excess of 80%, though there was no significant correlation between level of resistance of MRSA isolates and their respective protease, lipase or haemolysin production ($P>0.01$)

Aarestrup *et al,* (1999) reported that β-toxin was expressed by *S. aureus* in 72% of bovine, 11% of human carrier isolates and 13% of human septicemia isolates.

Goerke *et al.,* (2001) reported *hla*, the gene which encodes α-haemolysin has been implicated in virulence in an *in-vivo* guinea pig model of device related infection; levels of *hla* transcription were increased compared to levels of expression observed *in vitro.*

Coelho *et al.,* (2009) in their study reported, all haemolytic isolates presented beta-haemolysin, and 38% of the non-haemolytic isolates were able to express haemolysins in the presence of a beta-haemolytic strain.

Akinjogunla and Enabulele, (2010) compared haemolysin production between *S. aureus* and

CONS spp. with 16 (38.1%), 22 (52.4%) and 4 (9.5%) of *S. aureus* producing alpha (diffuse haemolysis), beta (clear haemolysis) and gamma (absence of haemolysis), respectively, while CONS spp**.** produced more of beta haemolysis than both alpha and gamma.

de Almeida *et al.,* (2013) reported that the frequency of *hla* genes among the isolates of northeast Brazil was high- 77.3%, and all *S. aureus* isolates from clinical mastitis presented the combination of the genes encoding for α-toxin and LUKE-D leukocidin. The *hlb* gene, which encodes β-haemolysin, was identified only in isolates from subclinical mastitis, accounting for 27.5% of total isolates.

2.9.3. Superantigens/ Enterotoxins as a virulent factor:

Superantigens interfere with the immune response by interfering with T-cells. Normally on the detection of an infectious substance T-cells are activated stimulating an immune response (Kohge *et al.,* 1998). Superantigens include the staphylococcal enterotoxins (SEs, also referred to as endotoxins) SEA-E and SEG-Q, and two toxic shock syndrome toxins, TSST1 and TSST2 (Orwin *et al.,* 2003).

Various enterotoxins, namely, *sea, seb, sec, sed, see, seh, seg, sei, sej* and *tsst-1*, have been reported among the *S. aureus* populations isolated from bovines. All the isolates tested failed to amplify the tsst-1 gene, although its prevalence in bovine isolates has been reported in association with sec and sed (Akineden *et al.,* 2001; Stephan *et al.,* 2001; Salasia *et al*., 2004).

S. aureus produces SEs within the temperature range of 10–48°C, with an optimum of 40–45°C. As the temperature decreases, the level of SE production also decreases. However, SEs remain stable under frozen storage. SEs are extremely resistant to heating and can survive the process used to sterilise low acid canned foods. SE production can occur in a pH range of 4.5–9.6, with an optimum of 7–8. Production of SE can occur in both anaerobic and aerobic environments; however, toxin production is optimum in aerobic conditions (ICMSF, 1996; Stewart, 2003).

In addition to their superantigen activity, Le Loir *et al.,* (2003) reported that SEs are also highly heat stable proteins that resist enzymatic digestion in the gut, and as a result are responsible for *S. aureus* food poisoning outbreaks.

Naffa *et al.,* (2006) studied genetic diversity of Jordanian clinical *S. aureus* and found Twenty-three isolates (23 %) were potentially enterotoxigenic among 100 isolates using multiplex PCR.The prevalence of *sea*, *sec* and (*sea+sec*) among the total clinical isolates was 15, 4 and 4%, respectively among all the isolates.

In a recent study by Zhang *et al.,* (2007), *S. aureus* from food , food poisoning and inpatients, positive detection ratio of multi-enterotoxin genes and newly found enterotoxin genes in inpatients' isolates were higher than the other two ($P<0.01$). *seg, sei, sek, seq* genes were mainly presented in isolates from inpatients while, *sea, see, she, seq* genes mainly presented in isolates from food samples and *sea, sep, sec* genes mainly found in strains from food poisoning. The most important fact of their study was that percentages of isolates from inpatient resistant to oxacillin were 71.43%, which was significantly higher than the percentages (2.33% and 2.38%) of the other two original isolates. The similarity among these isolates was above 70% and PFGE was agreed to be a useful discriminating typing method for *S. aureus* isolates.

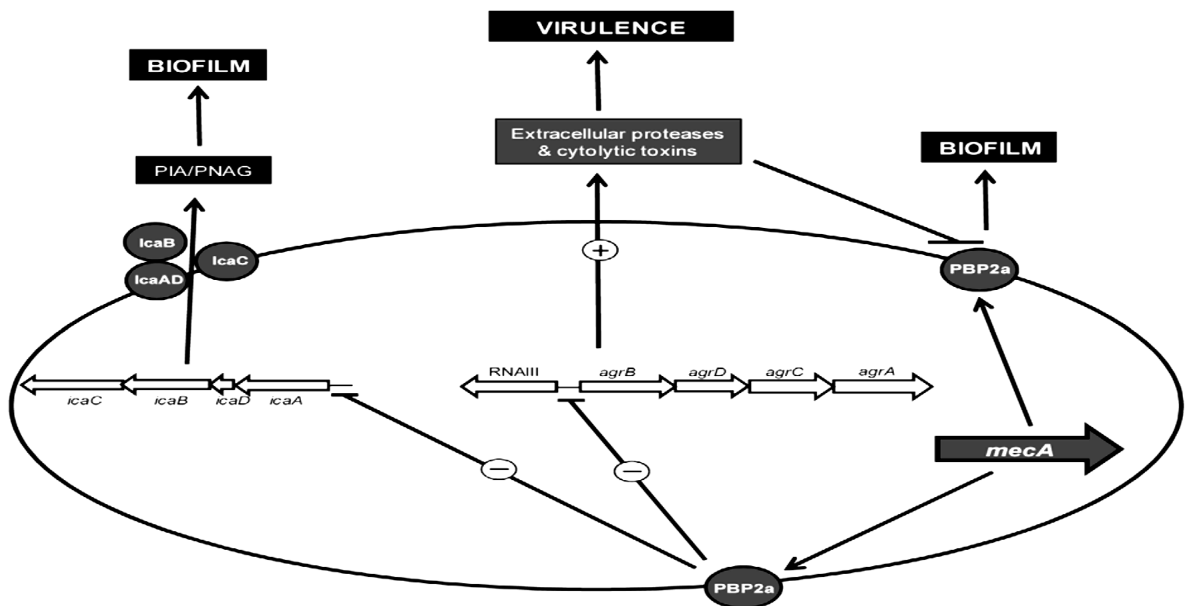

Figure 2.5. Model of PBP2a-mediated modulation of biofilm expression and virulence in *S. aureus*. High level PBP2a expression and homogeneous methicillin resistance results in repression of the *icaA*DBC and agr loci blocking PNAG production and reducing expression of extracellular proteases and cytolytic toxins. Reduced levels of extracellular protease and toxin production in turn correlate with PBP2a-promoted biofilm development and attenuated virulence (Pozzi *et al.,*2012).

Argudin *et al.,* (2010) suggested that *Staphylococcal* food poisoning is an intoxication that is caused by the ingestion of food containing pre-formed Staphylococcal Enterotoxin (SE). SEs are produced during the exponential phase of *S. aureus* growth, with the quantity being strain dependent. They also found that SEs are resistant to the heat and low pH conditions that easily destroy *S. aureus* bacteria. They also reported that the SEs are also resistant to proteolytic enzymes, hence SEs retain their activity in the gastrointestinal tract after ingestion. SEs range in size from 22–28 kDa and contain a highly flexible disulphide loop at the top of the N-terminal domain that is required for stable conformation and is associated with the ability of the SE to induce vomiting.

In a study by Kumar *et al.,* (2011) the proportions of toxin genes, namely, *hlb*, *seb, sec, sed*, *seg* and *sei,* in the isolates were found to be 94.3, 0.9, 8.4, 0.9, 10.2 and 49.5%, respectively while the proportions of agr genes I, II, III and IV were found to be 39.2, 27.1, 21.5 and 12.1%, respectively.

2.9.4. Extracellular enzymes as virulence factors:

Staphylococci mainly the species *S. aureus* are known to produce several extracellular proteases serine, cycteine and metalloenzymes. These proteases are important in the pathogenity processes of the bacteria (Maeda, 1996).

S. aureus secrete a range of extracellular proteases, which function as degradative compounds for redundant staphylococcal proteins; however they have also been linked to degradation of molecules involved in the host immune response (Lindsay, 2008).

Lipases secreted by *S. aureus* have been observed to increase resistance to phagocytotic killing. Furthermore, lipase interferes with the phagocytosis of the infectious lipase-producing *S. aureus* cells by host granulocytes, thus indicating a direct involvement of lipase in pathogenesis (Rollof *et al.,* 1988).

In previous studies, Gundgon and Devran, (2010) found that *S. aureus* isolates from raw meat samples were not proteolytic and lipolytic at +4 °C. They also found that 96.2 % , 89.1 % and 75.0 % of the *S. aureus* isolates obtained from meat, meatball and chicken samples showed proteolytic activity at +20 °C. Few of the isolates obtained from chicken samples (22.8 %) showed lipolytic activity under psychrotrophic conditions (+20 °C).

Rodrigues *et al.,* (2014) in his study found that the production of lipase varied among the isolates according to their source. They reported 86% of the isolates from human wounds were positive for lipase production. The frequencies of isolates positive for lipase production were 33.3% from cow udders, 15.4% from the nasal cavities of cattle, 82.9% from ricotta cheeses, and

100% and 91.7% from meat- and vegetable-contact surfaces, respectively.

In a study by Gundogan *et al.,* (2013), the frequency of positive protease and lipase production for *S. aureus* isolates were 23.5 and 11.8%, respectively from 34 coagulase-positive *Staphylococci* (CPS) isolated from raw calf meat (minced), chicken drumsticks, raw milk, ice cream and white cheese samples. Pro-teolytic and lipolytic activities were not found in the other CONS species.

El-Gayar *et al.,* (2014) found no significant relation between level of resistance of MRSA isolates and the protease production among 48 MRSA isolates. They categorized MRSA isolates into three groups depending upon the protease production as; strong protease producers (21%), intermediate protease producers (69%) and weak protease producers (10%).