Chapter 3

REVIEW OF LITERATURE

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The genus *Staphylococcus* belongs to the Phylum *Firmicutes* and family *Micrococcaceae* and consists of several species which occurs as human pathogens and commensals. The genus *Staphylococcus* has been classified into 32 species and 15 subspecies based on its biochemical characteristics and cell-wall composition. The important coagulase positive Staphylococcal species as human pathogen include the *Staphylococcus aureus* and coagulase negative Staphylococci include *S.epidermidis, S.saprophyticus and S.hemolyticus* [15, 16].

1. STAPHYLOCOCCUS AUREUS

1.1. Morphology

Staphylococci are Gram positive cocci, non-motile, non-sporing and arranged in grape-like clusters and few strains have microscopically visible capsules.

1.2. Cultural Characteristics

Strains of *Staphylococcus aureus* are aerobes or facultative anaerobes. They are non-fastidious and hence grow in ordinary media producing golden yellow non diffusible pigmented colonies. On 5 % sheep blood agar *S.aureus* produces beta hemolytic colonies and in MacConkey's medium it forms tiny pink color colonies due to the fermentation of lactose. One of the selective media to isolate *S.aureus* from mixed cultures is the mannitol salt agar containing 8-10 % NaCl.

1.3. Biochemical Characteristics

Sugars fermented by *S.aureus* include fructose, galactose, glucose, lactose, maltose, mannitol, mannose and ribose. Fermentation of mannitol has got diagnostic value in differentiating *S.aureus* from other species. *S.aureus* is positive for catalase, coagulase, phosphatase, liquefy gelatin, produce thermostable DNAase and reduce tellurite to metallic tellurium[16].

2. VIRULENCE PROPERTIES

2.1. Capsular Polysaccharide

A few strains of *S.aureus* produce the capsular polysaccharide which inhibits phagocytosis. Capsules help the organisms to adhere to prosthetic devices. Based on the immunology of capsules, *S. aureus* were serotyped into 11 types. Among these, types 5 and 8 have major clinical significance. Type 5 capsular polysaccharide is one of the most important virulence factors and is responsible for Staphylococcal bacteraemia and endocarditis. They also confer oxacillin resistance. Type 8 capsular polysaccharide induces the production of toxic shock syndrome toxin. These capsules can be electron microscopically viewed in catheters and intravenous lines[15].

2.2. Peptidoglycan and Teichoic acid

Both peptidoglycan and teichoic acid provide a sequence of biological activities and contributes to virulence. The biological activities include activation of complement pathway, enhance chemotaxis, induce the release of interleukin- 1 by monocytic cells and initiate opsonization with the help of opsonic antibodies.

Teichoic acids help the organisms to get adhered to the mucosal surfaces. Other important binding proteins like fibronectins, collagen and clumping factors are covalently linked to the peptidoglycan layer.

2.3. Protein A

Protein A helps the organism to escape opsonization and phagocytosis. Protein A also activates the complement components and triggers immediate and delayed type of hypersensitivity reactions. Protein A is immunogenic and is employed in co-agglutination technique that helps in the identification of gonococci, to serogroup Streptococci and to identify various bacterial antigens from sterile body fluids in diagnostic laboratories.

2.4. Enzymes

2.4.1. Catalase

Catalase is an important enzyme that helps the organism to escape from the toxic oxygen intermediates like hydrogen peroxide and reactive oxygen species synthesized in the myeloperoxidase- phagocytic system.

2.4.2. Clumping factor

It is present in the peptidoglyacan layer of *S.aureus* cells and they help the organism to bind to fibrinogen. Clumping factor can be detected by slide coagulase test and it is independent of coagulase reacting factor.

2.4.3. Coagulase

It is an exo-enzyme excreted free in the surrounding medium. Coagulase along with coagulase reacting factor (CRF) which is present in human or rabbit plasma in turn binds to prothrombin and converts fibrinogen to fibrin. The fibrin which coats the bacterial cells helps the bacteria to escape from phagocytosis and opsonization. About eight different types of coagulase enzyme have been identified. This free coagulase can be identified using tube coagulase test and is the important criteria to identify *S.aureus* from other species of *Staphylococci*.

2.4.4. Fibrinolysin

Fibrinolysin helps the organism to break the fibrin barrier and make them invade the host tissues.

2.4.5. Hyaluronidase

Hyalurodinase helps the organism to hydrolyze the mucopolysaccharide matrix in the host tissue and spread across the tissues.

2.4.6. Lipase

Lipases help the organism to spread into the cutaneous and subcuataneous tissues. Strains that cause severe furunculosis are found to be abundant producers of lipases.

2.4.7. Phosphotidylinositol- specific Phospholipase C

This enzyme is commonly detected in patients suffering from respiratory distress syndrome and disseminated intravascular coagulation. Those tissues damaged by this enzyme are prone to further destruction by complement components.

2.4.8. Nucleases and Phosphodiesterase

Both the enzymes have both exonuclease and endonuclease activity.

2.4.9. Beta-lactamases

Strains producing this enzyme destroy the beta-lactam antibiotics and make them ineffective. The genes for these enzymes are encoded in the plasmids and can be transferred to other bacteria by the process of conjugation, transformation or transduction. These enzymes can be inducible or constitutive.

2.5. Toxins

2.5.1. Alpha hemolysin

Alpha hemolysin has various toxic effects on host cells including the erythrocytes and polymorpho leucocytes. It is a protein of 33 kDa and is excreted into the surrounding medium. Once the alpha hemolysin binds to the host cells it forms a heptamer with a central pore. The heptameric alpha hemolysin pore opens up and causes the rapid efflux of potassium ions and influx of sodium and calcium ions resulting in rupture and osmotic swelling of the target host cells. This is an important dermonecrotic toxin and contributes the major pathogenic property in *S.aureus*

infection. It is also neurotoxic and produces a zone of clearing around the *S.aureus* colony when grown in medium containing 5 % sheep blood agar.

2.5.2. Beta hemolysin

Beta hemolysin is a sphingomyelinase and has a molecular weight of 35 kDa secreted into the surrounding medium. Hemolysis depends on the substrate constituents mainly sphingomyelin and lysophosphotidyl choline. It produces hot-cold type of hemolysis i.e., hemolysis on 5 % sheep blood agar can be enhanced by exposing the red blood cells to cold temperatures (4^0 C). The toxin initially destroys the red cell membrane and this is further enhanced when the temperature is lowered. This toxin along with the CAMP factor produced by *Group B Streptococci* produces synergistic hemolysis in 5 % sheep blood agar and helps in the presumptive identification of *Group B Streptococci*.

2.5.3. Delta hemolysin

Delta hemolysin destroys a wide range of target cells and has a molecular weight of 3 kDa. This exotoxin is produced almost by all strains of *S.aureus* and some coagulase negative *S.aureus*. It acts as a surfactant and destroys the cell membrane resulting in the leakage of cell contents.

2.5.4. Gamma hemolysin

The gamma hemolysin has three proteins which along with the two proteins Panton-Valentine – Leucocidin (PVL) form six “two component toxins” combinations. The five proteins do not confer any biological activity by themselves but six - two component toxin combinations has varying degrees of hemolytic

activity. They are leucocidal, destroy the polymorphoneutrophils and initiate degranulation, swelling and lysis.

2.5.5. Exfoliative toxin

Exfoliative toxins also caused epidermolytic toxins has a molecular weight of 24 kDa. It consists of two proteins exfoliative toxins A and B which are immunologically distinct but biologically similar. The proteolytic activity of the toxin results in the solubilization of epidermal mucopolysaccharide which further results in the splitting of the intracellular linkages with the stratum granulosum. Either of these toxin types can result in scalded skin syndrome.

2.5.6. Enterotoxins

Staphylococcal enterotoxins A, B, C, D, E, H and I cause *Staphylococcal* food poisoning. Ingestion of these preformed toxins results in vomiting and with or without diarrhea within 2 to 8 hours and is self-limited requiring only supportive therapy. Inflammation is appreciated all through the gastrointestinal tract.

2.5.7. Toxic Shock Syndrome toxin (TSST)

It causes toxic shock syndrome which is a multisystem disorder that will be presented with fever, vomiting, hypotension, mucosal hyperemia and is accompanied with erythematous rash that desquamates .TSST-1 is an important type that is responsible to initiate toxic shock syndrome. This toxin was first identified in the year 1978 in adolescents who used vaginal tampoons. This syndrome is also common in patients with skin & mucosal infections and also associated with surgical site infections.

Both Staphylococcal enterotoxins and TSST-1 serve as superantigens. They cause T-cell proliferation polyclonally and stimulate the T-lymphocytes abundantly regardless of antigenic specificity. This dysregulation of immune response result in enormous release of interleukins 1 & 2, tumour necrosis factor and gamma interferon which is responsible for multisystem disorder in case of toxic shock syndrome and staphylococcal food poisoning.

2.6. Slime formation

Some strains of *S.aureus* produce a diffuse labile exopolysaccharide layer called the slime. The slime layer helps the bacteria in colonization and to establish infection. Excess amount of this slime is produced by coagulase negative *Staphylococci* and is responsible for bacteraemia, catheter and prosthesis associated sepsis [17].

2.7. Biofilms

Biofilms are slime- like glycocalyx matrix in which microcolonies of *S.aureus* are embedded and these biofilms will adhere to biotic or abiotic surfaces. The biofilm matrix consists of polysaccharide, teichoic acids, extracellular DNA and various proteins produced by strains of *S.aureus* producing biofilms. The polysaccharide intracellular adhesin (PIA) present in the glycocalyx is β- 1,6-linked N-acetyl glucosamine residues and non- N- acetylated D- glucosamine residues. The PIA is an important mechanism contributing to biofilm mechanism. Some extracellular proteins are also responsible for biofilm formation which helps in the accumulation of antibiotic degrading enzymes which in turn responsible for adaptive antibiotic resistance[18].

3. REGULATION OF VIRULENCE FACTORS

The six regulatory loci responsible to control the functioning of virulence factors include accessory gene regulator *(agr*), Staphylococcal accessory regulator A *(sar A), sar H1, S.aureus* exoprotein expression *(sae),* repressor of toxins *(rot)* and extracellular protein regulator (*xpr).*

3.1. Accessory gene regulator

The *accessory gene regulator* is one of the global regulon and is a quorum sensing system that helps in the upregulation of exoprotein genes like hemolysins, toxic shock syndrome toxin, lipases, proteases and some membrane proteins. But it downregulates the expression of membrane associated proteins like fibronectin and Protein A.

The accessory gene regulator is a 3 kb locus and has two promotors P2 and P3. The P2 operon consists of four genes *AgrBDCA* and promoter P3 controls the expression of RNA III. The *agr D* codes for the autoinducing peptide precursor (AIP). The pro AIP is then secreted by the *agr B* membrane protein. The mature AIP binds to the *agrC* membrane sensor which in turn regulates *agrA*. Now *agrA* upregulates the transcription process of promotors P2 and P3 that initiates the production of RNA III effector molecule. RNA III is now responsible for the transcription of several exoprotein genes and repression of surface protein genes. The RNA III is also an mRNA coding for delta hemolysin gene. Hence reduction in delta hemolysin predicts some mutation or defect in *agr* loci. Based on the hypervariability regions in the *agr* loci it is classified into four types *agr I,II,III* and *IV*.

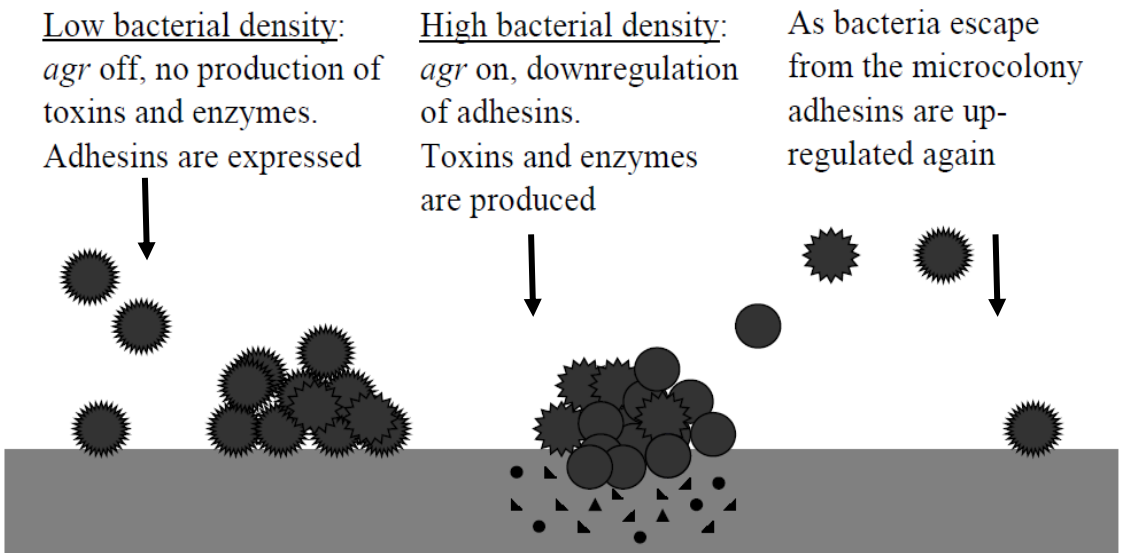

Fig :1 Agr- Quorum sensing system

The *accessory gene regulator* is a quorum sensing system and hence it gets activated when a quorum of bacteria is present and this is guided by a pheromone, autoinducing peptide produced by the *agr* operon (fig: 1).

When the concentration of bacterial population is low the *agr* system will not be activated and hence cell surface proteins like fibronectin binding protein and collagen binding protein will be initiated and help the organisms to undergo the process of adhesion and thus the colonization of the bacteria will be established. Once the colonization is established, bacterial concentration increases and this will consequently activate the *agr* system which inturn activates the expression of extracellular toxins and enzymes which progress tissue damage and represses the regulation of surface adhesion molecules like fibronectin-binding protein, collagen-binding protein, etc [19, 20].

3.2. Accessory gene regulator and biofilm formation

Agr locus down regulates the biofilm formation with the help of phenol soluble modulins (PSM) which behaves like a surfactant in dispersing the biofilm forming molecules. Therefore, active *agr* locus results in decreased biofilm formation. But PSM mutants or *agr* mutants favor increased or intact biofilm formation.

The disease causing and biofilm producing capacities of each *agr* class are different. *Agr* class I is often associated with endocarditis and superficial infections, *agr* class II and III are usually isolated from endocarditis and mostly nasal colonizers. *Agr* class IV is associated with exfoliative syndromes and mortality rate is higher with *agr* type II infections[18].

4. DISEASES CAUSED BY *S.AUREUS*

4.1. Folliculitis

Folliculitis is a benign symptom that is restricted only to the ostia of the hair follicle. It is characterized by red and painful lesion and do not exhibit any systemic manifestations

4.2. Furuncles and carbuncles

Furuncles are pyodermas that are usually deep seated will be red, painful lesions with necrotic centre. Carbuncles are also deep seated pyodermas that usually involve the subcutaneous tissues and the lesions coalesce with the formation of sinus tracts. They usually follow systemic manifestations.

4.3. Impetigo

Impetigo is one of the superficial lesions which are common among children and usually occurring in the exposed part of the body like face. They often proceed with the macule then transforming into a vesicle containing serosanguinous fluid. The vesicles will rupture forming a dry scab with erythematous margin. About 80-90% of impetigo is caused by *S.aureus* and the rest by *Group A Streptococci*.

4.4. Hydradenitis Suppurativa

It is characterized by multiple furuncles like lesions and associated with blocked apocrine sweat glands. It is predominantly present in the intertriginous areas of the body and usually presented as painful lesions without any systemic involvement.

4.5. Mastitis

It is the breast infection of lactating mothers which is usually presented as edematic, erythematic and firm swelling of the breast. Superficial breast abscess can be drained assisted by needle aspiration whereas the deep seated abscess can be removed by incisional drainage.

4.6. Wound Infections

Post surgical wound infections are usually caused by *S.aureus*. They are usually presented with red, painful lesions filled with serosanguinous fluid.

4.7. Bacteraemia and endocarditis

They usually occur due some underlying localized lesion or direct inoculation of the organism into the bloodstream through indwelling catheters or devices. They can be manifested as hemorrhagic lesions with chills and rigor. Endocarditis will be in a subtle manner and involve multiple systems

4.8. Meningitis and Pericarditis

Meningitis can result due to bacteraemia and pericarditis which can be manifested as a complication of endocarditis.

4.9. Staphylococcal superantigens

TSST-1 and enterotoxins exhibit superantigenic effect resulting in the onset of acute symptoms. Acute respiratory distress syndrome and disseminated intravascular coagulation are the common complications of TSS.

5. IDENTIFICATION OF *S.AUREUS*

5.1. Gram stain: *S.aureus* occurs as Gram positive cocci in clusters.

5.2. Cultural Characteristics:

5.2.1. Nutrient agar: *S.aureus* on nutrient agar grows as large, convex, smooth, shiny and emulsifiable golden yellow pigmented colonies.

5.2.2. Blood agar: On 5 % sheep blood agar *S.aureus* produces beta- hemolytic colonies.

5.2.3. MacConkey agar: On MacConkey's medium *S.aureus* produces tiny pink colonies due to lactose fermentation.

5.3. Catalase test: *S.aureus* produces catalase, an enzyme that is produced by aerobic organism to neutralize the toxic forms of oxygen metabolites. The presence of this enzyme can be detected by using 3% hydrogen peroxide. When the bacterial colony is emulsified in 3% H_2O_2 and if the organism produces catalase enzyme, it breaks down H_2O_2 into water and oxygen which is observed as effervescence.

5.4. Coagulase test: Coagulase enzyme produced by *S.aureus* can be detected by tube coagulase test. About 0.1 ml of the bacterial suspension is added to 0.5ml of human or rabbit plasma. The tubes were incubated at 37 ^{0}C for 3 to 6 hours and examined for any clot formation.

5.5. Mannitol Motility Test: *S.aureus* ferments mannitol and remains non-motile.

6. ANTIBIOTIC RESISTANCE IN *S.AUREUS*

6.1. Penicillin resistance

6.1.1. History and epidemiology of Penicillin resistant strains of *S.aureus*

In the pre-antibiotic era the mortality rate due to *S.aureus* bacteraemia increased to nearly 80% due to metastatic infections. With the development of penicillin in the year 1940, the prognosis of *S.aureus* infections improved. First incident of penicillin resistance developed in 1942 and these resistant strains subsequently started to spread across the hospitals and community establishing a new

wave of antimicrobial resistance. Bondi and Dietz first studied the role of penicillinase enzyme which is responsible for the degradation of penicillin[24]

6.1.2. Mechanism of Penicillin resistance

Penicillin resistance is conferred by a beta-lactamase enzyme, penicillinase. The gene for this beta-lactamase is present in transposable element present in the plasmid.

Beta-lactamase is an extracellular enzyme which will be synthesized when the strains of *Staphylococci* are exposed to beta-lactam antibiotics present in the medium. The enzyme hydrolyzes the beta-lactam ring and make the beta-lactam antibiotic inactive.

The gene that is responsible for this resistance is blaZ gene which is under the control of two antirepressor genes, blaR1 and *blaI*. For the synthesis of beta-lactamase enzyme it requires a sequential cleavage of these regulatory genes *blaR1* and *blaI*.

6.1.3. Detection of penicillinase producing S.aureus strains

6.1.3.1. Zone Edge test:

Penicillinase producing *S.aureus* strains can be detected using zone edge test. Synthesis of beta lactamase by the strains was evaluated by the presence of inhibitory zone around the penicillin disk. The strains were considered to produce beta-lactamase if the edge of the zone of inhibition is presented like a “cliff” and considered negative if the edge looks fuzzy like a “beach”[25].

6.1.3.2. Nitrocefin test:

Beta-lactamase production can also be detected by using disks that are impregnated with nitrocefin a chromogenic cephaloaporin. This chromogenic radical will change its color when the beta-lactam ring is hydrolysed by the action of beta-lactamase. Presence of beta-lactamase is indicated by the appearance of red color and negative reaction is by the absence of color change[25].

6.2. Methicillin Resistance in *S.aureus* (MRSA)

6.2.1. History and epidemiology of MRSA

The first penicillinase resistant semisynthetic penicillin was introduced in the year 1961 which was rapidly followed by the emergence of methicillin resistant *S.aureus*. This emergence created a major havoc among clinicians as treatment outcome will be worse with infections caused by these strains. Previously the dominant MRSA clones frequently caused nosocomial infections. However, recently these strains have started to disseminate in the community settings. The mortality rate has been increased among patients with community acquired MRSA infections (CA-MRSA). The presence of enterotoxin and panton-valentine genes has also added to the patient's morbidity with CA-MRSA infections.

6.2.2. Mechanism of Methicillin Resistance

The gene that is responsible for methicillin resistance is the *mecA* gene which is a mobile genetic element. This gene is a part of the genomic island termed as the Staphylococcal cassette chromosome (*SCCmec*) and four different types of *SCCmec* have been identified[26].

The *mecA* gene encodes the penicillin binding protein 2a *(PBP2a)*, a 78 kDa protein. The pencillin binding proteins are membrane-bound transpeptidase enzyme that is responsible for the transpeptidation reaction which cross- links the peptidoglycan chains. In contrast to the normal *PBP'*s the *PBP2a* substitutes have low affinity towards the beta-lactam drugs which results in the survival of *S.aureus* strains in high concentration of beta-lactam drugs. Methicillin resistance strains will confer resistance to all the group of beta-lactam drugs, including cephalosporins.

The mobile genetic element *SCCmec* is highly diverse and is integrated in the *S.aureus* genome at *attB* integration site and *orfX* gene is present at the 3' end. The *SCCmec's* contain two recombinases namely *ccrA* and *ccrB* that are responsible for integration and excision from the chromosome and thus makes the *SCCmec* as mobile genetic element. Two types of *ccr* complexes exists which include the *ccrA* and *ccrB* type. Based on its sequence variation there are different allotypes. *SCCmec* typing is employed for epidemiological surveillance of MRSA strains [27].

6.2.3. Community Acquired and Hospital Acquired MRSA (CAMRSA and HAMRSA)

CA-MRSA is very common in young and healthy individuals. The infection by CAMRSA will be manifested in the skin as boils and pimples. This is transmitted among individuals by skin to skin contact or acquired from contaminated surfaces and also common in crowded places with poor hygienic conditions.

Treatment includes local incision and drainage of lesions and can be treated with doxycycline or clindamycin. These isolates usually carry the Panton-Valentine gene and carry the *SCCmec* type IV genetic element. The usual practices to prevent

the spread of CAMRSA are to follow good personal hygiene, proper hand washing protocols and to allow the cuts and wounds to dry and covered.

HA-MRSA infections usually occur in individuals who have been recently hospitalized or with long term intensive facilities in hospitals. These HAMRSA strains usually cause bacteraemia, surgical site infections and resulting in infections at the site of implants.

These strains can be easily disseminated in the hospital environment due to poor hand hygiene, contaminated equipment and people with compromised immune system easily acquire the infection. These strains usually carry diverse types of the mobile genetic elements *SCCmec.*

Local lesions can be treated with surgical debridement whereas systemic infections like pneumonia & bacteraemia need hospitalization. The first line drug to treat HAMRSA infections includes parenteral vancomycin and other agents like co-trimoxazole, teicoplanin and linezolid can be administered. Gentamicin and rifampicin can be given as synergistic treatment.

Inorder to prevent the disseminating strains in the hospital, proper hand washing practices, hospital surveillance programs and all health personnel has to be educated [28].

6.2.4. Detection of MRSA strains

6.2.4.1. Oxacillin screen agar

Bacterial inoculum adjusted to 0.5 McFarland turbidity is spot inoculated (10µl) in Muller Hinton agar containing 4% NaCl and 6 µg oxacillin. The plates after

overnight incubation at 35^0 C were looked for any growth. Growth indicates that the strains were resistant to methicillin [29,30].

6.2.4.2. Cefoxitin disc diffusion method

Cefoxitin remains as the surrogate marker to detect the *mecA* mediated methicillin resistance among S.aureus strains. Cefoxitin induces the expression of *mecA* gene. If the strains tested with 30µg cefoxitin disc give a zone of inhibition ≤ 21mm will be considered as resistant to methicillin[30].

6.2.4.3. CHROM agar

This chromogenic medium is developed for the identification of MRSA. Strains growing as green colonies were considered to be MRSA [31].

6.2.4.4. MecA PCR

Though a sequence of phenotypic methods are available to detect the MRSA strains the current gold standard for the detection of these strains remains the *mecA* PCR using specific primers.

6.3. Quinolone resistance

6.3.1. History and epidemiology of Quinolone resistance

In 1980's fluroquinolones were initially used to treat Gram negative infections. However these drugs were used to *S.aureus* infections. The use of these drugs to treat *S.aureus* infections has been dramatically reduced due to emerging resistance. Antibiotic selective pressure in the hospital setting is one important reason for the rapid emergence of quinolone resistant strains.

6.3.2. Mechanism of Quinolone resistance

Quinolone resistance is due to the spontaneous mutation in the topoisomerase IV or DNA gyrase or due to existence of multidrug efflux pumps. It has been proved that when *S.aureus* strains present in the mucosal surfaces or external nares are exposed to suboptimal concentration of quinolones which was used to treat Gram negative infections, due to antibiotic selective pressure quinolone resistant *S.aureus* strains emerge and get disseminated in the hospital environment.

6.4. Macrolide resistance in *S.aureus*

Various macrolide resistance mechanisms exist in *S.aureus* strains. The genes responsible for macrolide resistance include *ermA* and *ermC* that cause 23SrRNA methylation and thus preventing the ribosomal binding of the drug. These genes can be expressed constitutively of inducibly.

The ribosomal methylation can result in cross resistance to Macrolides, Lincosamides and Streptogramin B and hence referred ad MLS_B phenotype. The mechanism of macrolide resistance is the existence of macrolide efflux pumps which is encoded by the *msrA* gene. These efflux pumps confer resistance to macrolides containing 14- 15 membered ring and referred as M-phenotype[31].

Clindamycin resistance can exists as both inducible and constitutive type which is coded by *erm* genes. The inducible resistant strains will be difficult to be detected in the laboratory as they appear sensitive to clindamycin and resistant to erythromycin when they are not placed together. In this case, clindamycin will not work invivo and lead to therapeutic failures.

In case of efflux mechanism which is coded by *mrsA* gene, the *S.aureus* strains will be susceptible to clindamycin and resistant to erythromycin invivo and invitro and the strain will not become resistant to clindamycin during therapy [32].

6.4.1. Detection of macrolide resistance

MS phenotype in *S.aureus* strains can be detected in strains showing resistance to erythromycin having zone diameter ≤ 13 mm and sensitive to clindamycin having zone diameter ≥ 21mm.

iMLS$_B$ phenotype are strains showing erythromycin resistance having zone diameter ≤ 13mm and sensitive to clindamycin giving a zone diameter of ≥ 21mm . They show a D- shaped zone around the clindamycin disc and flattens towards erythromycin disc

cMLS$_B$ phenotype show resistance to both erythromycin and clindamycin having zone diameters ≤ 13mm and ≤ 14 mm respectively.

6.5. Mupirocin resistance

Decolonization of MRSA is usually done by intranasal application of Mupirocin. This drug has been established as one of the effective topical antibacterial agents for the nasal decolonization of *S.aureus* strains, since the 1980 s.

Mupirocin also called Pseudomonic acid A, is an antimicrobial agent that competitively inhibits the synthesis of *isoleucyl tRNA synthetase*. Since nasal colonization of MRSA plays an important role in nosocomial infection, increased use of mupirocin for nasal decolonization has resulted in the emergence of Mupirocin resistant isolates.

There exist two types of mupirocin resistance - Low level and high level mupirocin resistance. Low level mupirocin resistance is due to point mutations that occur in the gene coding for *tRNA* synthetase, the native gene *ileS-1*. Low level mupirocin resistant (LLMR) isolates usually have a MIC range between 8-256 µg/ml.

High level mupirocin resistance (HLMR) is due to a gene encoded in a plasmid called *Mup A* gene also called as *ileS2*, which encodes an additional modified *isoleucyl t-RNA synthetase*. HLMR strains have a MIC range ≥ 512 µg/ml.

Treatment of HLMR strains with mupirocin remains ineffective. In case of LLMR strains, treatment with mupirocin can be done by slightly increasing the concentration but this can also predict treatment failures.

6.6. Vancomycin resistance

Vancomycin has gained more importance in clinical settings because it remains as one of major drug to treat MRSA infections. Overuse of vancomycin has paved way for the emergence of vancomycin resistant strains or strains possessing reduced susceptibility to vancomycin.

6.6.1. History of Vancomycin resistance

MRSA strains first emerged in the 1960's and few years back in 1950's vancomycin was discovered by Eli Lilly after a visit by a missionary who sent a sample of dirt from which the organism *Amycolatopsis orientalis* was isolated .This organisms produced a substance that inhibited the growth of Gram positive organism. Since it was brown in color it was termed as "Mississippi mud" and this drug was approved by Food and Drug administration in the year 1958.

6.6.2. Mechanism of action of Vancomycin

Vancomycin binds to the C- terminal *D-Ala-D-Ala* residues present in the precursor of peptidoglycan and it result in the formation of a stable-noncovalent complex which in turn results in the non-availability of the precursor for cell-wall synthesis. Vancomycin mainly inhibits the late-stage peptidoglycan biosynthesis. Any simple mechanism that prevents the binding of vancomycin to *D-Ala-D-Ala* residues will make the drug ineffective.

The cell wall synthesis takes place in the septum that is dividing bacterial cell and does not involve the whole cell membrane. This suggests that vancomycin has to diffuse to the tip of the dividing septum and to get bound to the peptidoglycan precursors. The distance that vancomycin need to travel depends on the length of the septum and length will be longer in the later part of the dividing cycle.

6.6.3. Vancomycin Resistant *Staphylococcus aureus*

Vancomycin Resistant Enterococci (VRE) emerged in the year 1980's. High level or complete vancomycin resistance in *S.aureus* is due to *VanA* gene acquired from VRE whereas reduced vancomycin susceptibility is not *VanA* mediated. *VanA* mediated resistance in *S.aureus* was first reported in the year 2002 in a patient from Michigan.

The *VanA* sequence was similar to that of the *VanA* sequence of *Enterococcus faecalis*. In *S.aureus VanA* gene was encoded in a transposon present in a plasmid. The transposon is *Tn1546* that harbors the *VanA* gene which confers vancomycin resistance in *Enterococci*. These strains have vancomycin MIC range of ≥ 16 μg/ml.

6.6.4. Vancomycin Intermediate *Staphylococcus aureus*

The basic mechanism behind vancomycin intermediate resistance is thickened cell-wall and vancomycin binds to non-vital targets present in the peptidoglycan layer. Glycopeptides usually binds to the murein monomers in the cytoplasmic membrane and thus peptidoglycan synthesis is completely inhibited. But to get bound to these targets they need to travel through nearly 20 peptidoglycan layers without being trapped by the first targets, the *D-ala-D-ala* residues present in the completed peptidoglycan layers or the nascent peptidoglycan layer.

Since there exists a lot of *D-ala-D-ala* targets in the peptidoglycan layer, vancomycin gets trapped in the peptidoglycan layers. Thus there will be some decrease in the drug potency. Therefore if the concentration of *S.aureus* is high, then major molecules of vancomycin gets adsorbed to the cell wall and thus the tissue concentration of the drug becomes low and reaches a lower therapeutic concentration than required. Thus it has been overcome by decreasing the concentration of bacteria by incisional drainage of the abscess.

The first VISA strain was isolated by Hiramatsu in the year 1997 from a 4 month old patient who has undergone cardiac surgery in Japan and named as Mu50.

Transmission electron microscopy and biochemical analysis clearly depicted that this strain had several layers of peptidoglycan. Because of these peptidoglycan layers more vancomycin molecules are trapped before reaching the target, the murein monomers present in the cytoplasmic membrane and large concentration of vancomycin is needed to saturate the murein monomers. This type of mechanism is termed as “affinity trapping” process.

Few research work suggests, the thick peptidoglycan is destroyed by the trapped vancomycin molecules this further preventing the entry of vancomycin molecules into the inner part of the cell-wall. This phenomenon is termed as "clogging phenomenon".

The VISA strains synthesize an abnormal non-amidated murein monomer that has a greater affinity towards vancomycin when compared to the normal murein monomer which further enhances the trapping and clogging phenomenon.

Another mechanism of reduced susceptibility is in reduction of peptidoglycan turnover. New layers of peptidoglycan formed will displace the older ones and this is done with the help of autolytic enzymes. VISA strains show a reduction in autolytic activity. VISA strains usually have a vancomycin MIC range of 4-8 µg/ml[33].

6.6.5. heteroresistant Vancomycin Intermediate *Staphylococcus aureus* (hVISA)

hVISA strains will have vancomycin MIC in the susceptible range of ≤ 2 µg/ml. But there exists a population of cells in the intermediate range. These intermediate resistant strains will be present at a frequency of 10^{-5} to 10^{-6} cells. Hence routine CLSI methods fail to detect these isolates. Population analysis Profile Area Under the curve method remains as the gold standard method for detecting these strains. The hVISA (Mu3) strain was first isolated by Hiramatsu in the year 1997 from the sputum of a 64 year old patient presented with MRSA pneumonia.

These hVISA strains can also be detected among methicillin sensitive S.aureus (MSSA) having vancomycin MIC's <0.5 µg/ml[2]. A case report from

Argentina 2011, has described vancomycin treatment failure in an acute infection caused by *Methicillin Sensitive Staphylococcus aureus* (MSSA). Another study by Jain Hu et.al, gives a prevalence rate of hVISA among MSSA strains as 4.1%. This arises the need to screen these hVISA population among MSSA strains having MIC's ≤ 1.5 μg/ml [34].

6.6.6. Vancomycin MIC creep

Treatment failures have become common with infections caused by strains having MIC in the susceptible range. The term "MIC creep" is commonly used to demonstrate the changing trend of vancomycin MIC over a period of time[2].

Table: 1 Global scenario of hVISA/VISA

Study	Place of Study	Year of study	No. of isolates	% of hVISA
Hiramatsu *et al.,*	Japan	1998	129	9.3%
Liu C. *et al.,*	Review study	1997-2001	7920	1.69%
Iyer *et al.,*	India	2008	50	2%
Van Hal SJ *et al.,*	Australia	2009	417	12%
Calin Liu *et al.,*	China	2015	184	22%
Maj Puneet *et al.,*	Maharastra India	2011- 2013	475	6.9%
C N Chaudhari *et al.,*	AFMC, Pune, India	2015	58	7%
Sibabrata *et al.,*	Tripura, India	2015	100	6%
Devi *et al.,*	CMC Vellore ,India Review study	2015	--	--
Auttawit *et al.,*	Thailand	2016	82	34%
A.Singh *et al.,*	UP, India	2017	79	36%

6.6.7. Phenotypic features of hVISA/VISA

6.6.7.1. Cell wall changes

One of the consistent features among hVISA/VISA strains include the thickened cell wall that can be demonstrated by Transmission Electron Microscopy (TEM).

6.6.7.2. Reduction in autolytic activity

Reduced autolytic activity is also a common feature of hVISA/VISA strains. Studies suggest that altered peptidoglycan hydrolase (murein hydrolase) activity is responsible for reduced autolytic activity. Any mutation in accessory gene regulator (*agr*) can result in decrease in the synthesis of murein hydrolase [35]. Reduction in autolytic activity can be detected by Triton X-100 induced lysis method.

6.6.7.3. Colony morphology of hVISA

hVISA strains grow as small colony variants and usually present as mixed colony morphology. They grow slowly forming a mixture of small and large colonies. This highlights the importance to check vancomycin susceptibility with colonies of different morphotypes. The pigment production will also be reduced in these isolates[2].

6.6.7.4. Accessory gene regulator and hVISA/VISA

Studies reveal that agr dysfunction is associated with vancomycin intermediate resistance. Initially it appeared that *agr type II* specificity is associated with VISA/hVISA phenotype. But recent studies reveal that hVISA/ VISA phenotype were over presented with *agr* type I specificity. Also those strains that belong to all

the *agr* types can develop intermediate resistance to vancomycin on constant exposure to the drug.

Since *agr* serves as the global regulon for most of the virulence factors, particularly the exotoxins, the hVISA/VISA strains with reduced *agr* activity show reduction in the synthesis of exotoxins. Loss of *agr* function is represented by reduced autolytic activity and reduced susceptibility to platelet microbicidal protein.

6.6.7.5. hVISA / VISA - Agr dysfunction- colony spreading- biofilm formation

The *agr* deficient phenotype of *S.aureus* will exhibit enhanced biofilm formation both invitro and invivo. Intact *agr* system is also required for bacterial colony spreading that can be demonstrated in soft agar medium [13].

6.6.8. Genetic mechanisms – hVISA/VISA

Various genetic mechanisms lie behind the regulation of vancomycin intermediate resistance. Few mechanisms include the mutation of regulatory genes, *vraSR*, a two component regulatory system, *walkR* which is also a two component regulatory system and *rpoB* mutation. Also, mutation of another regulatory gene *graR* along with *rpoB* mutation will convert low-level vancomycin resistance to high level vancomycin resistance [36,37].

6.6.9. Clinical infections and hVISA

hVISA/VISA strains are often associated with infections with high bacterial load. These include bacteraemia, pneumonia, endocarditis and patients with orthopaedic implants, artificial valves and pacemakers. Conditions usually get worse

with the commencement of vancomycin therapy. Inorder to decrease the bacterial load surgical debridement is the better option as it brings down the bacterial load.

6.6.10. Alternate therapy for hVISA/VISA infections

Rifampicin and fusidic acid can be administered non-parenterally for the treatment of multidrug resistant MRSA. Linezolid, a oxazolidinone is used to treat hVISA/VISA infections mainly endocarditis. The only disadvantage behind this drug is its toxicity. Daptomycin, a cyclic lipopeptide can be used for treating hVISA/VISA infections but the bacterial load should be low. Therefore it should be used with caution to treat bacterial endocarditis. This drug effect is promising when it is used along with trimethoprim- sulfamethoxazole.

Other drugs include Quinupristin- dalfopristin and tigecycline. They have exhibited a better invitro activity against hVISA/VISA infections.

Newer antimicrobials like dalbavancin, oritavancin, telavancin which are semisynthetic lipoglycopeptide gives promising effects against hVISA/VISA infections. Newer cephalosporins like ceftaroline and ceftobiprole also possess good invitro activity against hVISA/VISA strains.

6.6.11. Laboratory detection of hVISA/VISA strains

6.6.11.1. Screening method:

Screening of hVISA/VISA strains can be done using brain heart infusion agar containing 6 µg vancomycin. In this method 10 µl 0.5 MacFarland bacterial

suspension is spot inoculated and after incubation presence of any single bacterial colony was noted.

6.6.11.2. E-test macromethod:

This test is performed using 2.0 MacFarland bacterial suspension. Vancomycin and teicoplanin E-strips were used. The bacterial strains were considered heteroresistant if the MIC's for vancomycin and teicoplanin is ≥8 μg/ml or teicoplanin MIC ≥12 μg/ml regardless of vancomycin MIC.

6.6.11.3. E-test GRD (Glycopeptide resistance detection)

This test is performed using a double-sided predefined gradient of both teicoplanin and vancomycin. After inoculation with 0.5 MacFarland suspension and incubation, the test is indicated as positive for hVISA if the MIC was ≥ 8 μg/ml for either of vancomycin or teicoplanin.

6.6.11.4. Standard E-test :

This test is done using gradient strips individually for both vancomycin and teicoplanin. About 0.5 Macfarland bacterial suspension is used as inoculum. The MIC results are interpreted accaording to CLSI guidelines.

6.6.11.5. Population Analysis Profile Under Curve method:

This is the gold standard confirmatory method for the detection of hVISA and VISA strains. This test is performed according to Wootten et al using two standard strains Mu50 and Mu3. Isolates were defined as hVISA if the AUC ratio of the test strain to that of the Mu3 strain is 0.90 – 1.29.

7. Treatment of *S.aureus* infection

7.1. Penicillin resistant *S.aureus*

Only rare strains of *S.aureus* remain susceptible to penicillin and others remain resistant. Penicillin resistant *S.aureus* is due to the production of the enzyme penicillinase. Inorder to treat the infections caused by penicillin resistant strains, beta lactamase inhibitor like amoxicillin-clavulanate and ampicillin sulbactam can be used. The alternate option is to use a penicillase- resistant penicillin like oxacillin or nafcillin. Other antibiotics to treat Methicillin susceptible *S.aureus* include lincomycin, erythromycin and clindamycin. There also exist erythromycin and clindamycin strains which can be detected by D-test.

7.2. Treatment of Methicillin resistant *S.aureus*

Methicillin resistant *S.aureus* carry the *mecA* gene that encodes for a modified penicillin binding protein , *PBP2a*, a modified transpeptidase enzyme that has low affinity towards beta lactam antibiotics and hence become resistant to antibiotics like methicillin, oxacillin, nafcillin and other cephalosporins [21,22].

Most of the nosocomial MRSA strains remain multidrug resistant and thus patients should be treated with parenteral vancomycin or can be treated with teicoplanin if the patient is allergic to vancomycin[23]. Community acquired MRSA remain susceptible to other antibiotics like tetracycline, minocycline, doxycycline, and co-trimoxazole.

7.3. Treatment of hVISA/VISA and VRSA strains

These strains evolve in patients receiving long term vancomycin therapy or receiving vancomycin in suboptimal concentration for a long period of time. These patients can be treated with newer antimicrobials like linezolid, quinupristin/dalfopristin, daptomycin, dalbavancin and oritavancin.