2. REVIEW OF LITERATURE

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile spherical bacteria that occurs in microscopic clusters resembling grapes (staphylo-). It is often referred to as "*S. aureus*" or "Staph aureus" in medical literature. *Staphylococcus* was first identified in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses in a knee joint **(Barber and Rozwadowska-Dowzenko, 1948)**. It belongs to the family *Staphylococcaceae* which is nosocomial pathogen capable of causing a wide range of human diseases. *Staphylococcus* is among top five most common causes of infections occurring after any injury or surgery in humans (**Ogston, 1984; Rayan, 2004)**. Approximately 30 different species of Staphylococci can infect humans, but most infections are caused by *Staphylococcus aureus*. It is expected that 20% of the human population are long-term carriers of *S. aureus* which can be found usually in the nose, back of the throat and on the skin of healthy adult hospital workers. Moreover due to its ability to affect a wide range of species, it can be easily transmitted from one species to another particularly between humans and animals. Under normal circumstances bacteria do not cause disease, but damage of the skin or other injury may allow the bacteria to defeat the natural protective mechanisms of the body, leading to infection **(Kluytmans *et al.,* 1997)**. The most common diseases caused by *Staphylococcus aureus* include furuncles, carbuncles, impetigo, abscesses, septicemia, necrotizing pneumonia, and catheter-induced infective endocarditis, atherosclerosis. *S. aureus* uses large number of virulence factors to accommodate a diversity of niches in its human host. Apart from the classical appearances of *S.aureus*-induced diseases, the pathogen also invades and survives within mammalian host cells. The survival tactics of the pathogen are as diverse as strains or host cell types used (**Fraunholz and Sinha 2012**).

2.1.1 Colonization and disease

S. aureus is both a commensal organism and a pathogen. It is estimated that 20% of individuals are constantly nasally colonized with *S. aureus*, and 30% are intermittently colonized. Nevertheless, various other sites may be colonized, including the axillae, groin, and gastrointestinal tract. Colonization faciliatates a reservoir from which bacteria can be introduced when host defenses are breached, whether by shaving, aspiration, insertion of an indwelling catheter, or surgery. Colonization

undoubtedly increases the risk for consequent infection (**Kluytmans *et al.*, 1997; Wertheim *et al.*, 2005; Gordon and Lowy, 2008**). It has been reported that blood isolates were indistinguishable to nasal isolates in 82% of patients in bacteremia (**Von Eiff *et al.*, 2001**). Colonization also permits *S. aureus* to be transmitted among individuals in both health care and community settings. The basis for *S. aureus* colonization is complex and incompletely understood but appears to involve the host's contact with *S. aureus* and the ability of *S. aureus* to adhere to host cells and to evade the immune response (**Wertheim *et al.*, 2005**).

2.1.2 Virulence factors and disease

The armamentarium of virulence factors of *S. aureus* is widespread, with both structural and secreted products playing a role in the pathogenesis of infection. Two remarkable features of staphylococci are that a virulence factor may have several functions in pathogenesis and that multiple virulence factors may perform the same function. In causing an infection, *S. aureus* has several surface proteins, called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs), that mediate adherence to host tissues (**Gordon and Lowy, 2008**). MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component. MSCRAMMs appear to play a crucial role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections. Various *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections (**Patti *et al.*, 1994; Foster and Hook, 1998**).

Once *S. aureus* adheres to host tissues or prosthetic materials, it is able to grow and persist in various ways. *S. aureus* can form biofilms (slime) on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials (**Tung *et al.*, 2000**). The capability to form and reside in biofilms is one reason why prosthetic-device infections, for example, can be so difficult to eradicate without removal of the device. In vitro, *S. aureus* can also invade and survive inside epithelial cells, including endothelial cells, which theoretically may also allow it to escape host defenses, particularly in endocarditis (**Ogawa *et al.*, 1985; Moreillon *et al.*, 2000**). *S. aureus* is also able to form small-colony variants (SCVs), which may contribute to persistent and recurrent infection. In vitro, SCVs are able to "hide" in host cells without causing significant host-cell damage and are relatively protected from

antibiotics and host defenses. They can later revert to the more virulent wildtype phenotype, possibly resulting in recurrent infection (**Proctor *et al.*, 1995**).

S. aureus has many other characteristics that help it evade the host immune system during an infection (**Foster, 2005**). Its most important defense is production of an antiphagocytic microcapsule. The zwitterionic capsule can also induce abscess formation (**Tzianabos *et al.*, 2001**). *S. aureus* may also secrete chemotaxis inhibitory protein of staphylococci or the extracellular adherence protein, which interfere with neutrophil extravasation and chemotaxis to the site of infection (**Foster, 2005**).

In addition, *S. aureus* produces leukocidins that cause leukocyte destruction by the formation of pores in the cell membrane (**Gladstone and VanHeyningen, 1957**).During infection, *S. aureus* produces numerous enzymes, such as proteases, lipases, and elastases, that enable it to invade and destroy host tissues and metastasize to other sites. *S. aureus* is also capable of producing septic shock. It does this by interacting with and activating the host immune system and coagulation pathways (**Lowy, 1998; Gordon and Lowy, 2008**).

2.1.3 *S. aureus* carriage

In 1932 the Norwegian dermatologist and Niels Danbolt reported that 22 out of 24 patients with recurring furunculosis had staphylococci with the same biochemical properties both in the nose and in the lesions. Therefore, he suggested that the nasal 'infection' was responsible for the recurrent skin infection demonstrating autoinfection. In 1948, Moss *et al.* demonstrated that skin carriage of *S. aureus* was dependent on nasal carriage in patients with normal skin. Local penicillin treatment, could reduce nasal *S. aureus* carriage from 97% to 37% and simultaneously reduce skin carriage from 57% to 38% after 15 days (**Moss *et al.*, 1948**). They recommended that the nasal vestibule was the primary site of *S. aureus* carriage. Further investigations have confirmed that the most frequent site of carriage is the anterior nares of the nose (**Williams, 1963**). In addition to above studies, Cole *et al.* identified the moist squamous epithelium on the septum adjacent to the nasal ostium to be the main colonisation site in carriers (**Cole *et al.*, 2001**). In the common adult population, *S. aureus* can also commonly be found at other body sites such as the axillae (8%), chest/abdomen (15%), perineum (22%), intestine (17–31%) (**Williams, 1963**), and vagina (5%) (**Guinan *et al.*, 1982**). Interestingly, recent reports have shown higher carriage rates in the throat than in the nares when sampled in parallel. In addition, an intestinal carriage of 20% in healthy individuals has been reported, and even though

nasal carriage may predispose to intestinal carriage, lone intestinal carriage was also detected (**Acton *et al.,* 2009**). In an MRSA screening performed at The University Hospital of Lausanne a total of 12456 individuals were sampled in the nose, groin and throat. The sensitivity of detection by culture was 48%, 63% and 61% for the three sampling sites, respectively. Combinations of two sites increased the sensitivity to 76–89% while inclusion of all three sites increased it to 96% (**Senn *et al.,* 2012**). These results suggested a broader preference of *S. aureus* for colonisation than previously defined, at least for MRSA (**Sollid *et al.,* 2013**).

2.1.4 Treatment and antibiotic resistance

The staphylococcal infections are generally treated by penicillin and its derivatives, and cephalosporins across the world. However, penicillin resistance is exceptionally widespread, and first-line therapy is most frequently a penicillinase-resistant β-lactam antibiotics (e.g. Oxacillin, cloxacillin, nafcillin). Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis, but its use is limited because of the high risk of damage to the kidneys **(Korzeniowski and Sande, 1982; Bayer *et al.,* 1998)**. The choice of drug and duration of treatment depends on the site of infection and on severity. Antibiotic resistance in *S. aureus* was infrequent when penicillin was first introduced in 1943. By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant; and, by 1960, this had risen to 80%. Today, *S. aureus* has become resistant to many commonly used antibiotics **(Chambers, 2001)**. It expresses resistance to beta-lactam antibiotics by beta-lactamase production, methicillin resistance, penicillin tolerance, and deficiency in penicillin binding proteins (PBPs). The clinical implication of all these phenomena is not fully defined **(Lacey, 1984)**.

Infections caused by antibiotic-resistant strains of *S. aureus* have arrived at epidemic ratios worldwide (**Grundmann *et al.,* 2006**). Emergence of antibiotic resistance by *S. aureus* can be visualized as a series of epidemic waves (**Figure 2.1**) (**Chambers and DeLeo, 2009**). The overall burden of staphylococcal disease caused by methicillin *resistant S. aureus* strains (MRSA) in particular, is increasing in many countries in both healthcare and community settings (**Kaplan *et al.,* 2005; Klevens *et al.,* 2007**). In the USA the emergence of community associated MRSA (CA-MRSA) strains as a major cause of skin and soft-tissue infections (**Fridkin *et al.,* 2005**) accounts for much of this increase. The rapidity and extent to which CA-MRSA strains have spread has been significant.

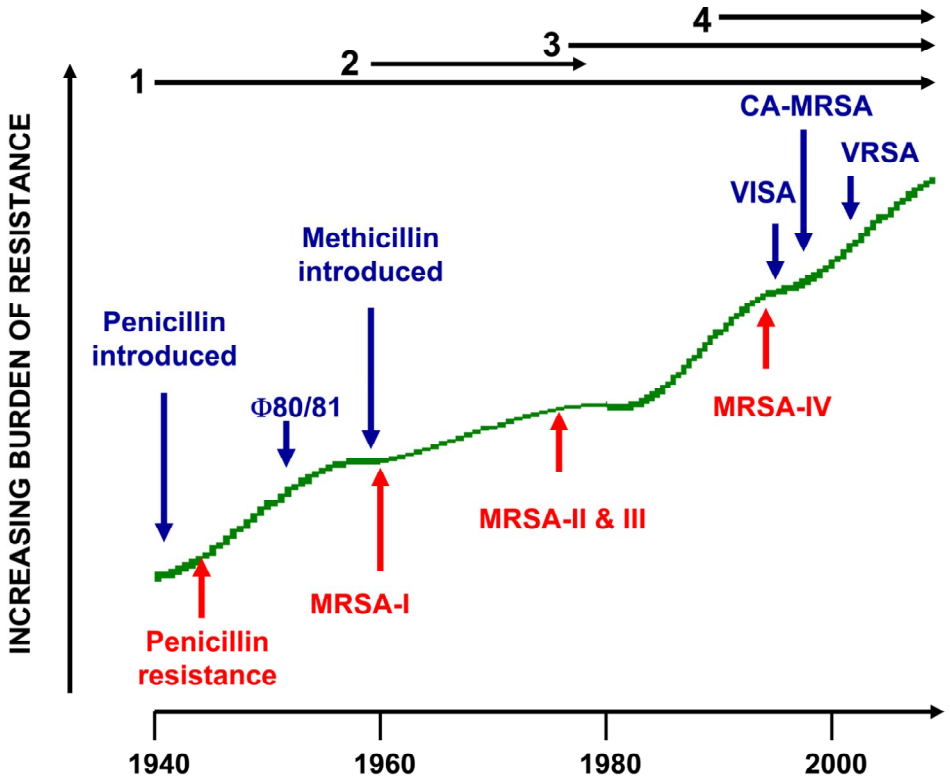

Figure 2.1: A timeline of the four waves of antibiotic resistance in *Staphylococcus aureus* (Chambers and DeLeo, 2009)

In addition to the USA, CA-MRSA strains have been reported from Canada, Asia, South America, Australia, and throughout Europe, and other countries with historically low prevalence of MRSA (**Vandenesch *et al.*, 2003; Laupland *et al.*, 2008; Nimmo and Coombs, 2008**). CA-MRSA strains have shown a significant diversity in the number of various clones that have been identified.

2.2 Methicillin resistant *Staphylococcus aureus* (MRSA)

Soon after the discovery of the penicillin by A. Flemming in 1928, a strain of *S. aureus* quickly emerged that secreted an enzyme called penicillinase, which hydrolyzes penicillin into inactive penicilloic acid. In the 1950s, scientists at a UK-based pharmaceutical company, Beecham, discovered that placing bulky substituents on the penicillin side chain would protect penicillins from penicillinase-mediated devastation due to steric hindrance (**Morell and Balkin, 2010**). Consequently, in 1959, Beecham introduced methicillin, a class of penicillinase resistant ß-lactams.

Unfortunately, however, the first cases of methicillin-resistant *Staphylococcus aureus* (MRSA) were reported in Europe just a few years later in 1961 (**Barber, 1961**). Since then MRSA has become the most frequent multidrug resistant pathogen causing nosocomial infections in humans and other mammals as well across the world. MRSA now accounts for over 60% of *S. aureus* isolates in U.S. intensive care units (**Boucher and Corey, 2008; Kluytmans and Struelens, 2009**). The MRSA strains are capable to grow up in the presence of methicillin, oxacillin and nafcillin antibiotics (**Zetola *et al.*, 2005**). Now a days, MRSA strains are the most common multidrug resistant pathogen causing nosocomial infections in not only in the hospitals across the world but also in communities, and are often resistant to several antibiotics (**Deurenberg and Stobberingh, 2009**). The infections caused by these strains are most frequent in patients in hospitals, nursing homes, and other intensive care units. There is an urgent need to combat the prevalence and epidemiology of MRSA infections and the relationship between susceptible and resistant strains. Since resistance development is an evolutionary process, continuous monitoring is essential to identify emerging pathogens at national and global levels, to create and refine strategies for controlling antimicrobial resistance and to guide clinical decisions regarding appropriate treatment (**Mostofsky *et al.*, 2011**).

The original MRSA infections associated with exposure in the health care setting, particularly in hospitals are referred to as hospital-acquired MRSA (HA-MRSA) **(Klein *et al.*, 2007)**. In 1990s, a new strain of MRSA emerged in the community setting occurring among young healthy individuals with no exposure to the healthcare setting. The infections caused by these strains are called community-acquired MRSA (CA-MRSA) **(Beam and Buckley, 2006; David and Daum, 2010)**. CA-MRSA is becoming a potential threat to the public-health. The new strains of *S. aureus* exhibiting unique permutations of virulence factors and resistance traits have been associated with high morbidity and mortality in the community. Outbreaks of epidemic furunculosis and cases of severe invasive pulmonary infections in young, otherwise healthy people have been particularly remarkable (**Zetola *et al.*, 2005**).

2.3 Community acquired-methicillin resistant *Staphylococcus aureus* (CA-MRSA)

2.3.1 Pathogenesis of CA-MRSA infection

The cases of MRSA have been reported in healthy community-dwelling individuals without established risk factors for MRSA infections because they are apparently acquired in the community, these infections are referred to as community-acquired (CA)-MRSA. The number of CA-MRSA infections appears to be increasing, and the strains responsible for these infections have now entered the health care setting, blurring the line between "community" and "hospital" strains (**Gonzalez *et al.*, 2006; Seybold *et al.*, 2006**). Skin and soft-tissue infections (SSTIs) are the most common type of CA-MRSA infection, contributing for around 90% of cases, of which 90% are abscesses and/or cellulitis with purulent drainage (**Fridkin *et al.*, 2005**). CA-MRSA strains also appear to be virulent in particular, with the capability to cause fulminant, irresistible infections, such as necrotizing fasciitis, necrotizing pneumonia, bone and joint infections accompanied by septic thromboembolic disease (**Gonzalez *et al.*, 2005a; Gonzalez *et al.*, 2005b; Miller *et al.*, 2005**), purpura fulminans with or without Waterhouse-Friderichsen syndrome (**Adem *et al.*, 2005**), orbital cellulitis and endophthalmitis (**Rutar *et al.*, 2006**), infections of the central nervous system (**Sifri *et al.*, 2007**) and bacteremia (**Seybold *et al.*, 2006**) and endocarditis (**Chua *et al.*, 2008**).

The strains that cause these virulent infections carry staphylococcal chromosome cassette *mec* type IV (SCC*mec*IV), the smallest of the SCCs that bestow methicillin resistance, and are usually susceptible to several non–β-lactam antibiotics. This is in contrast to the multidrug-resistant nosocomial MRSA strains that carry larger SCC*mec* types (**Zetola *et al.*, 2005**). CA-MRSA strains may also have a growth advantage over HA-MRSA strains (**Baba *et al.*, 2002**). Although SCC*mec*IV has appeared in several different genetic backgrounds, pulsed-field gel electrophoresis (PFGE) types USA300 and USA400 both *agr* type III—accounted for the vast majority of CA-MRSA infections in individuals without the usual MRSA risk factors or health care contact in the United States (**McDougal *et al.*, 2003**). USA300 is now the predominant strain of interest. Some of these USA300 isolates that cause infections are PVL (Panton-Valentine leukocidin) positive but methicillin susceptible (**Moran *et al.*, 2006**). The basis for the apparent increased virulence of CA-MRSA strains is not exactly known. Numerous factors have been proposed, such as increased

fitness, improved evasion of the host immune system, and unique toxin production. Since these strains usually contain PVL, which is usually absent in HA-MRSA strains, some scientists assume that this protein, with leukocytolytic and dermonecrotic activity, is responsible (**Gordon and Lowy, 2008**).

2.3.2 Epidemiology of CA-MRSA

Until the mid 1990s, MRSA rarely caused infections among community members without exposure to the health care setting. An outbreak of CA-MRSA infections happened between 1989 and 1991 among indigenous Australians in western Australia without health care contact (**Udo *et al.*, 1993**). It soon became obvious that these infections were due to the emergence of new, distinct strains of MRSA, now called CA-MRSA strains. CA-MRSA infections were also reported in people from neighboring regions. In the late 1990s, numerous cases of violent MRSA infection also occurred among individuals in the United States without established risk factors for MRSA (**Gordon and Lowy, 2008**). Four children died of CA-MRSA infections in Minnesota and North Dakota from 1997 to 1999. All the cases were swiftly fatal and were associated with necrotizing pneumonia or pulmonary abscesses and sepsis (**MMWR, 1999**). Earliest reported cases of CA-MRSA infection in the US were caused by a USA400 strain (MW2) (**CDC, 1999**). The strain responsible for these infections was ST1 and PFGE type USA400 (also known as the MW2 strain) (**McDougal *et al.*, 2003**). Subsequently, clonal outbreaks of skin and soft-tissue infection caused by CA-MRSA were also reported among prison inmates, men who have sex with men, soldiers, and athletes, particularly football players (**CDC, 2003; Kazakova *et al.*, 2005**). The most common disease appearance associated with CA-MRSA is infection of the skin and soft tissues (**Fridkin *et al.*, 2005**). Skin and soft tissue infections (SSTI) account for at least 90% of CA-MRSA infections. CA-MRSA SSTIs are usually moderately severe to severe. The cases of CA-MRSA skin infection and necrotizing pneumonia were reported internationally as well (**Lina *et al.*, 1999; Vandenesch *et al.*, 2003**). In addition to causing necrotizing pneumonia, CA-MRSA has recently been reported to cause infections or infectious complications in situations in which *S. aureus* or MRSA is an unusual pathogen (**Davis *et al.*, 2007**). These have included cases of necrotizing fasciitis caused by PFGE type USA300 (**Miller *et al.*, 2005**), as well as cases of pyomyositis (**Ruiz *et al.*, 2005**) purpura fulminans with toxic shock syndrome (**Kravitz *et al.*, 2005**), and Waterhouse-Friderichsen syndrome (**Adem *et al.*, 2005**). The epidemiology of CA-MRSA is quite similar regardless of

country of origin. Isolates tend not to be multiple drug-resistant, *SCCmec* types IV and V are typically present, and infections of skin and soft tissue are the most common. The presence of PVL among CA-MRSA isolates is more variable. Outbreaks and epidemics of CA-MRSA have now spread across the world with a similar epidemiology, even though the specific clones that have emerged vary with geographical location. CA-MRSA strains are not only escapees from healthcare facilities; their genotypes show that they are not closely related to endemic hospital clones and these community strains are susceptible to several antibiotics to which hospital strains are routinely resistant (**Chambers and DeLeo, 2009**).

The Methicillin resistant *S. aureus* (MRSA) strains continue to be a notorious pathogen for both hospital-associated as well as community-acquired infections in India. The MRSA infection has now become endemic in India. The incidence of MRSA varies from 25 per cent in western part of India (**Patel *et al.*, 2010**) to 50 per cent in South India (**Gopalakrishnan and Sureshkumar, 2010**). Community acquired MRSA (CA-MRSA) infections have been increasingly reported from India (**D'Souza *et al.*, 2010; INSAR, 2013**).

2.3.3 Virulence of CA-MRSA

The Community acquired-methicillin resistant *Staphylococcus aureus* (CA-MRSA) strains have the capability to infect otherwise healthy people, indicates increased virulence. **Voyich *et al.* (2005)** reported that the CA-MRSA strains MW2 are more virulent in a mouse infection model than common HA-MRSA strains such as COL and MRSA252. In addition, they found that the enhanced virulence of CA-MRSA strains was accompanied by enhanced survival in human neutrophils, suggesting that the interaction of the pathogen with neutrophils, a critical step in the establishment of S. aureus infection, determines the increased virulence of CA-MRSA compared to HA-MRSA (**Voyich *et al.*, 2005**).

The MW2 (also known as USA400) is a highly virulent CA-MRSA strain. This is noticeable not only in human disease but also in animal models (**Ward and Turner, 1980; Baba *et al.*, 2002**). In the begining, its only resistance genes were *mec* and *blaZ,* which encodes penicillinase. Scientists sequenced USA400 and compared its sequence with the sequences of 5 other strains (N315, a Japanese MRSA; Mu50, a vancomycin-resistant MRSA; E-MRSA-16, an epidemic MRSA in the United Kingdom; COL, a MRSA strain; and NCTC8325, a commonly used reference strain)

to identify potential virulence factors associated with this strain. USA400 was the only strain to contain the PVL operon. In addition, it contained 16 unique superantigen genes, including 11 exotoxin genes and 5 enterotoxin genes (**Gordon and Lowy, 2008**). These genes had at least a 2% difference in their amino acids, compared with their homologues. One exception was staphylococcal enterotoxin H (*seh*), which was unique to USA400 (**Baba *et al.*, 2002**) and can cause a toxic-shock–like syndrome (**Ren *et al.*, 1994**). USA400 also contained a novel gene cluster dubbed "bacteriocin of *S. aureus"* (*bsa*). *bsa* encodes a potential bacteriocin, or antibacterial agent. This bacteriocin could help USA400 compete with other colonizing flora and increase the chance of infection with this strain (**Baba *et al.*, 2002**). These data suggest that there are several factors that may contribute to the virulence of USA400 (MW2) and that these factors will be useful for future investigation (**Gordon and Lowy, 2008**).

2.3.4 Colonization and CA-MRSA

The anterior nares are the common reservoir for nosocomial *S. aureus* infections, including HA-MRSA. However, it has been reported that other sites of colonization or modes of transmission play a crucial and apparent role in the development of CA-MRSA infection (**Gordon and Lowy, 2008**). Heterosexual contact was recently identified as a mode of transmission of CA-MRSA. Most cases had genital CA-MRSA colonization without nasal colonization (**Cook *et al.*, 2007**). In an outbreak research of CA-MRSA abscesses among St. Louis Rams football players, no MRSA was isolated from nasal or environmental samples. possibly other sites of colonization, shared items, or an unsampled environmental site played a role in transmission (**Kazakova *et al.*, 2005**). Future epidemiological investigations of CA-MRSA should include sampling of several environmental and body sites in addition to the anterior nares (**Gordon and Lowy, 2008**).

2.3.5 Treatment and antibiotic resistance of CA-MRSA

The emergence of new strain of MRSA in the community (CA-MRSA) has apparently contributed more complications in the control and prevention of infections caused by the MRSA strains. The CA-MRSA strains possess entirely distinct genetic and phenotypic features from hospital acquired MRSA (HA-MRSA) strains (**Laupland *et al.*, 2008; Chen *et al.*, 2010**). Due to the differences in the structure of CA-MRSA and HA-MRSA strains, their antibiotic resistance patterns also differ which influence the hospital and community environments (**Martinez, 2008**). Rapid increase of

CA-MRSA strains isolated from the patients in different geographic locations across the world suggest that traditional nosocomial MRSA strains are being replaced by CA-MRSA which have profound impact on empirical therapy of suspected staphylococcal infection (**Popovich *et al.*, 2008**). Most of the beta-lactam antibiotics, fluoroquinolones and clindamycin are no longer effective for treating the range of staphylococcus infections especially skin and soft-tissue infections. In many regions where CA-MRSA skin and soft tissue infections are widespread, traditional antimicrobial treatment choices by fluoroquinolones and clindamycin are not justified, leaving physicians to depend on other feasible options with long-acting tetracyclines, trimethoprim-sulfamethoxazole (TMP-SMX), linezolid, vancomycin which have severe impact of harsh adverse effects **(Powell and Wenzel, 2008; Chambers and DeLeo, 2009)**. In addition, adjunctive agents such as rifampin and fusidic are used in combination with above antibiotics (**Nathwani *et al.*, 2008)**.

Surgical incision and drainage is the preferred method of treatment for cutaneous abscesses caused by CA-MRSA infections. Apart from this adjunctive antimicrobial therapy is of modest or no benefit in most of the cases (**Llera and Levy, 1985; Lee *et al.*, 2004; Moran *et al.*, 2006**). Antibiotic therapy after drainage of CA-MRSA abscesses is not normally suggested unless the patient has severe or widespread disease, or has rapid increase in the presence of associated cellulitis; has signs and symptoms of systemic illness; is very old or very young or has medical co-morbidities or immune compromised (e.g., HIV infections, neoplastic disease, diabetes mellitus); or has an abscess in an area that is difficult to drain or an abscess that is associated with septic phlebitis (**Gorwitz *et al.*, 2006**). Vancomycin is the preferred drug for treatment of serious MRSA infections. Nevertheless, prolonged, persistent, or recurrent bacteremia during therapy (**Khatib *et al.*, 2009**), high rates of microbiological and clinical failures (**Dombrowski and Winston, 2008**), nephrotoxicity (**Lodise *et al.*, 2008**), and increasing incidence of non-susceptible strains (**Steinkraus *et al.*, 2007**) restrict its effectiveness. Arbitrary clinical trials of alterative drugs such as linezolid and daptomycin demonstrate that they are comparable, or more precisely, non-inferior, but not superior, to standard therapy (**Arbeit *et al.*, 2004; Shorr *et al.*, 2005; Fowler *et al.*, 2006**) and drug toxicity remains a major cause of concern in spite of the choice of agent.

Some more drugs are in the development phases which are likely to become available for treatment of MRSA infections in the coming future (**Pan *et al.*, 2008**).

Telavancin, dalbavancin, and oritavancin are derivatives of vancomycin that kill *S. aureus* swiftly in a concentration-dependent manner *in vitro*. Whether more rapid killing will transform into improved efficacy over vancomycin for more serious infections, such as endocarditis or bacteremia, remains to be investigated (**Chambers and DeLeo, 2009**). Carbapenems and cephalosporins that bind PBP 2a, the penicillin-binding protein that mediates methicillin resistance, with much higher affinity than the currently available betalactams, have been developed (**Koga *et al.*, 2005**). Two cephalosporins, ceftobiprole and ceftaroline (**Anderson and Gums, 2008; Parish and Scheinfeld, 2008**), have been demonstrated to be clinically effective for the treatment of MRSA skin and soft tissue infections (SSTIs). A concern with these and the other anti-MRSA beta-lactams under development is that they are very broad spectrum antibiotics for the targeted treatment of MRSA infection. Further investigations are required to define their eventual role in therapy of MRSA infections. The vancomycin-derivatives and anti-MRSA beta-lactams, which can only be administered by intravenous route, do not account for orally active compounds. Different non-conventional approaches to treatment and prevention of MRSA infections have been or are under investigation (**Chambers and DeLeo, 2009**). The major challenges in the development of novel compounds include prohibitively expensive cost, potential for hypersensitivity with recurring administration, short half lives with systemic administration. It will take several years to come up with novel effective and safe anti-MRSA agents in the clinic. However, judicious use of drugs that are now available is crucial to avoid further erosion of the antimicrobial therapy of CA-MRSA (**Otto, 2010**).

2.4 *In silico* drug target identification

The rising incidence of nosocomial infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin- resistant *Enterococcus faecium* (VRE) and the threat that high-level vancomycin resistance will eventually spread to staphylococci highlight the need for vigilance in the unending war against pathogenic microbes (**Gold and Moellering, 1996; Salyers and Amabile-Cuevas, 1997**). At present commonly used antibiotics are targeted at a amazingly small number of vital cellular functions such as cell wall, DNA, RNA, and protein biosynthesis, and instances of resistance to these antibiotics are prevalent and well documented in literatures (**Swartz, 1994**). Therefore, there is an urgent need of novel antibiotics to

combat the increasing crisis of antibiotic-resistant bacteria, and targeting of new pathways will likely play a significant role in discovery of these new antibiotics. In fact, a number of vital cellular pathways, such as secretion, cell division, and many metabolic functions, remain untargeted today (**Moir *et al.*, 1999**).

The process of novel potential drug target identification has been revolutionized by technological advances which aid the drug discovery process. The increasing availability of large number of complete microbial genome sequences has provided an opportunity for researchers to explore all possible potential drug targets in pathogens which have not yet been discovered. The availability of high-quality genome-wide metabolic reconstructions (**Oberhardt *et al.*, 2009**) presents a new approach for the rational and systematic identification of metabolic drug targets in a pathogenic organism. Functionally, biological pathways can be categorized into three classes i.e. metabolic pathways, gene regulation pathways and signal transduction pathways. Metabolic pathways are accountable for the chemical alterations involved in the biosynthesis or decomposition of various metabolites (such as proteins, nucleic acids, lipids) (**Chavali *et al.*, 2012**). During the metabolic reconstruction process, there might be hundreds of putative metabolic enzymes whose structural and functional information is not yet identified by experimental methods. Annotation of proteins with metabolic information is mandatory for mapping the metabolic pathways on genomic data **(Freiberg**, **2001)**.

The biological function relies on the coordinated action of many molecular interactions of gene products and metabolites, systematically organized in so-called pathways. (**Persidis, 1998; Ideker *et al.*, 2001; Pawson and Nash, 2003**). Any disturbance in the pathway flow or connections through slight alteration of one or more individual pathway components can lead to impaired functions (**Brown and Superti-Furga, 2003**). One of the most important advantages of the metabolic pathway analysis in pathogens are identification of novel potential drug targets, which can be exploited for novel drug discovery to combat the infections caused by life threatening pathogens.

2.4.1 Genome annotation

In any metabolic reconstruction, there might be hundreds of putative metabolic enzymes with no experimentally identified function. These reactions and associated gene-protein reaction (GPR) relationships may be assembled strictly based on existing functional annotations of the genome or based on evidence from related organisms.

Even some very well characterized enzymes may have other unexpected activities. In such cases, misannotated enzymes may yield wrong model predictions leading to errors in drug targeting (**Chavali *et al.*, 2012**). For example, an enzyme may be mistakenly predicted to be essential if the activity of another enzyme, which is not included in the network, can account for the same function. As a result, this represents one important knowledge gap in the assembly of metabolic networks, and the inclusion of more refined enzyme annotations will directly improve drug target predictions (**Hsiao *et al.*, 2010; Szappanos *et al.*, 2011**).

2.4.2 Metabolic pathway mapping

The genome comparison tools have to account for the complex relationships that exist between protein families, which, in turn, help to understand the metabolic network of microbes (**Christoph, 2001**). The phylogenetic profiling approach enables the identification of groups of genes occurring in certain sets of bacteria that indicate that they are functionally coupled (**Marcotte *et al.*, 1999; Pellegrini *et al.*, 1999**). Such an approach is called as metabolic pathway mapping. A prerequisite for mapping metabolic pathways on genomic data is the annotation of proteins with metabolic information. The commonly known Enzyme Commission (EC) numbers have been given to characterized proteins in order to classify the enzymatic chemical reactions of proteins. Unfortunately, the EC system only classifies enzymatic proteins and does not describe the cellular role of a gene product. Therefore, different genome comparison databases include additional classification systems that explain the biological process (e.g. transcription, translation), pathway (e.g. fatty acid biosynthesis) or cellular location in addition to the molecular function of the protein (**Christoph, 2001**).

2.4.3 Gene essentiality analysis

The most common method to identify potential drug targets has been through the prediction of essential genes. The essential genes are those genes which are indispensible for the survival of organism. The enzymes encoded by essential genes are classically hypothesized as drug targets. Gene knockouts might lead to a redistribution of flux through the network if the perturbed gene or gene product affects the removal of a particular flux-carrying reaction (**Oberhardt *et al.*, 2009**). A gene-protein reaction (GPR) aids in mapping the effects of a genetic (or pharmacological) perturbation on the associated reactions, and thus the network. Gene-level perturbations that result in reduced or zero flux through a biomass reaction

correspond to growth-reducing or lethal gene knockouts, respectively (**Chavali *et al.*, 2012**). For instance, in a reconstruction of metabolic pathways *Mycobacterium tuberculosis*, five previously known drug targets were encoded by genes predicted to be essential from computational analysis (**Beste *et al.*, 2007**). For metabolic reconstructions of pathogens, enzymes that are predicted to be essential will provide new experimental hypotheses and opportunities for novel drug discovery.

2.5 Structure based drug design approaches in novel inhibitor designing

Traditional drug discovery and development process is a tedious, expensive, time-consuming and full of risk in particular. It is estimated that a drug from concept to its launch in the market would take approximately 12 years and cost more than US$800 million on an average (**DiMasi *et al.*, 2003**). Numerous new technologies have hence been developed and implemented in drug research and development to cut short the whole process and to trim down the expenses. Computer-aided drug design (CADD) is one of such promising technologies (**Jorgensen, 2004**). The concept of CADD has evolved very rapidly, especially in the recent decade as an unprecedented development of structural biology and computational technologies. CADD technologies including molecular modeling and simulation have become promising in drug discovery. Recently, CADD has even been used in designing highly selective ligands for a certain target that shares very similar structures with many proteins, which is difficult to be done by other methods. In the postgenomic era, owing to the remarkable increase of small molecule and biomacromolecule information, CADD tools have been applied in almost every phase of drug discovery process, greatly changing the strategy and pipeline for drug discovery (**Jorgensen, 2004; Tang *et al.*, 2006**). CADD approaches have contributed to the successful discovery of numerous novel enzyme inhibitors, including inhibitors of thymidylate synthase, HIV-1 Protease and purine nucleoside phosphorylase inhibitors. In each case, CADD was used to predict the binding affinity of an inhibitor designed from a lead compound *prior* to synthesis (**Reddy and Erion**, **1998**).

The process of structure-based drug design is an iterative one and often proceeds through multiple cycles before an optimized lead goes into phase I clinical trials. The first cycle includes the cloning, purification and structure determination of

the target protein or nucleic acid by one of three principal methods: X-ray crystallography, NMR, or homology modelling. Using computer algorithms, compounds or fragments of compounds from a database are positioned into a selected region of the structure. These compounds are scored and ranked based on their steric and electrostatic interactions with the target site and the best compounds are tested with biochemical assays. In the second cycle, structure determination of the target in complex with a promising lead from the first cycle, one with at least a micromolar inhibition in vitro, reveals sites on the compound that can be optimized to increase potency. Additional cycles include synthesis of the optimized lead, structure determination of the new target-lead complex, and further optimization of the lead compound. After several cycles of the drug design process, the optimized compounds usually show marked improvement in binding and, often, specificity for the target (**Anderson**, **2003**). The structure based drug design process generally involves following steps:

(i) Selection of a drug target

The choice of a drug target is primarily made on a pharmacological and biochemical basis. The ideal target macromolecule for structure-based drug design is one that is closely linked to human disease and binds a small molecule in order to carry out a physiological function. The target molecule usually has a well-defined binding pocket or active site (**Anderson**, **2003**).

(ii) Evaluating a target for structure-based drug design

Once a target has been identified, it is necessary to obtain accurate structural information. There are three primary methods for structure determination that are useful for drug design: X-ray crystallography, NMR, and homology modeling. Crystal structures are the most common source of structural information for drug design, since structures determined to high resolution may be available, and the method is useful for proteins that range in size from a few amino acids to 998 kDa. Typically, crystal structures determined with data extending to beyond 2.5 Å are acceptable for drug design purposes (**Nissen *et al.***, **2000**).

(iii) Active site identification in the target

Structure-based design begins with the identification of a potential ligand binding site (active site) on the target molecule. Ideally, the target site is a pocket or protuberance with a variety of potential hydrogen bond donors and acceptors, hydrophobic characteristics, and sizes of molecular surfaces. The ligand binding site can be the

active site, as in an enzyme, an assembly site with another macromolecule, or a communication site necessary in the mechanism of the molecule. The basic inputs for this step are the 3D structure of the protein and a pre-docked ligand in PDB format, as well as their atomic properties. Both ligand and protein atoms need to be classified and their atomic properties should be defined, basically, into four atomic types:

- **Hydrophobic atom**: all carbons in hydrocarbon chains or in aromatic groups.
- **H-bond donor**: Oxygen and nitrogen atoms bonded to hydrogen atom(s).
- **H-bond acceptor**: Oxygen and sp2 or sp hybridized nitrogen atoms with lone electron pair(s).
- **Polar atom**: Oxygen and nitrogen atoms that are neither H-bond donor nor H-bond acceptor, sulfur, phosphorus, halogen, metal and carbon atoms bonded to hetero-atom(s).

All aspects thought to be responsible for binding affinity and selectivity are collected. This knowledge is then exploited to create new ideas on ways to improve existing ligands or to develop new alternative bonding skeletons for a target (**Gerhard**, **2000**).

(iv) Drug Design Methods

Once the three dimensional structure of the target is evaluated and the active site in the structure is identified, there are several paths to developing a good lead based on structure of the target. Current methods for structure-based drug design can be divided roughly into two categories.

1. Virtual Screening
2. *De novo* ligand designing

The first category is about "finding" ligands for a given receptor, which is usually referred as database searching (**Meng *et al***, **1992**). In this case, a large number of molecules are screened to find those fitting the binding pocket (active site) of the receptor. Some researchers call this "virtual screening" in analogy to the bioassay screening procedure employed in the traditional drug discovery process. The earliest programs for performing 3D database searching are DOCK (**Schoichet and Kuntz**, **1993**), AUTODOCK (**Morris *et al,* 1998**) which are freely available for non-commercial use to researchers.

Another category of structure-based drug design methods is about "building" ligands, which is usually referred as *de novo* design. In this case, ligand molecules are built up within the constraints of the binding pocket by assembling small pieces in a stepwise

manner. These pieces can be either atoms or fragments (**Wang *et al.,* 2000)**. These techniques are raising much enthusiasm in the drug design community.

2.6 Fructose-bisphosphate aldolase (FBA) as a potential therapeutic drug target

Fructose-bisphosphate aldolase (FBA) enzyme is encoded by **fbaA** gene. Fructose-bisphosphate aldolase (FBA) class II is found in pathogenic bacteria and fungi, such as *Staphylococcus aureus, Helicobacter pylori, Mycobacterium tuberculosis, M. pneumoniae, M. leprae, Yersinia pestis, Candida albicans*, which is distinct from the class I present in mammals. FBA has been reported as potential therapeutic drug target in *Escherichia coli* (**Singer *et al.,* 1991**), *Bacillus subtilis* (**Fonvielle *et al.,* 2004**) *Candida albicans* (**Rodaki *et al.,* 2006**) and *Mycobacterium tuberculosis* (**Pegan *et al.,* 2009**). These FBAs are involved in second reversible step of the glycolytic pathway, which supplies glyceraldehyde 3-phosphate for downstream enzymes in the pathway and fructose 1,6-bisphosphate (FBP) for gluconeogenesis. Together, the substrates and products of the FBA reaction are crucial for the supply of these precursor molecules to other biochemical pathways essential for the survival of pathogenic bacteria. This enzyme is involved in several metabolic pathways of microbes such as Glycolysis, Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, and Methane metabolism (**Yadav *et al.,* 2012**). Inhibiting this enzyme by novel inhibitor(s) can obstruct several pathways such as Glycolysis, Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism, and Methane metabolism, which might prove fatal for the community acquired-methicillin resistant *Staphylococcus aureus* (CA-MRSA).