Chapter I

Background and significance of the study
*Staphylococcus* is a highly versatile and constantly evolving human pathogen, causing hospital- and community-associated infections worldwide. It continues to fascinate and bother microbiologists with its impeccable adaptive nature and ability to evade human immune system. The remarkable success of this bacterium as a nosocomial pathogen is attributed to its commensal nature, an array of toxin genes and its extraordinary ability to acquire resistance to newer antibiotics (Uhlemann et al., 2014). *Staphylococcus* is a genus of Gram-positive bacteria having a wider host range and diverse life style. It comprises more than 40 different species which include coagulase positive staphylococci such as *Staphylococcus aureus* and coagulase negative staphylococci (CoNS) such as *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus* etc. *S. aureus* is a common colonizer of anterior nares, throat, gut, groin, skin folds and mucosa of 30% of human population. Infections are usually caused by the colonizing strain when natural barriers are breached. Thus carriers are at a higher risk of infections under immunocompromised or hospitalized conditions. The mode of transmission is primarily by direct contact with an infected person or contaminated objects. Staphylococci are capable of causing a spectrum of infections ranging from minor skin and soft tissue infections such as cellulitis, impetigo, furuncle to highly invasive diseases including bacteremia, endocarditis, sepsis, osteomyelitis, ventilator-associated pneumonia etc. It is also one of the major pathogens associated with surgical site infections.

1.1 Staphylococci in the antibiotic era

Staphylococcal infections in hospitalized patients have been a great challenge ever since the discovery of this pathogen. Control and prevention of these infections are complicated by the frequent emergence of drug resistant strains. The mortality rate associated with the infections caused by staphylococci was about 80% before the introduction of penicillin into clinical practice in the early 1940s (Skinner and Keefer, 1941). This miracle drug turned out to be a failure with the emergence of beta-lactamase producing strains in hospitals within a decade. These strains which caused infections in both hospital and community largely belonged to the phage type 80/81, which disappeared on introduction of methicillin in 1960. Methicillin, a beta-lactamase stable
beta-lactam also met with a similar fate, with staphylococci acquiring the \textit{mecA} gene, which confers resistance to methicillin. The \textit{mecA} gene which is carried on a mobile genetic element, namely Staphylococcal Cassette Chromosome \textit{mec} (SCC\textit{mec}) codes for an altered penicillin binding protein, PBP2a with reduced affinity to beta-lactams. In contrast to beta-lactamase mediated resistance, methicillin-resistance leaves the entire beta-lactam class of antibiotics ineffective. Methicillin resistant \textit{S. aureus} (MRSA) soon became an established nosocomial pathogen and continues to be a burden in hospital settings across the globe. MRSA is troublesome, especially in intensive care units (ICUs) where carriers are at a higher risk of blood stream infections. Various clones of hospital acquired MRSA (HA-MRSA) evolved over time and the evolution of MRSA was also accompanied by the acquisition of resistance to non-beta-lactam antibiotics, challenging the therapeutic options for MRSA treatments.

MRSA clones exhibit striking differences with respect to fitness, virulence traits and antibiotic resistance pattern on account of their polyphyletic origin, i.e., MRSA clones emerged from genetically distinct lineages of methicillin susceptible \textit{S. aureus} (MSSA), rather than from a single ancestral clone (Diep et al., 2006). It is also important to note that only a handful of MRSA lineages have succeeded in spreading globally and causing epidemic waves, whereas MSSA is highly diverse, with several clones circulating internationally (Rolain et al., 2015). The genetic backgrounds of the successful MRSA clones were often similar to that of the epidemic lineages of MSSA, suggesting that epidemicity preceded antibiotic resistance. Point mutations in housekeeping genes (\textit{arcC, yqiL, gmK} etc.) and the acquisition of various SCC\textit{mec} elements have been the key events in the evolution of new lineages of MRSA (fig 1.1). The epidemiology of MRSA has been extremely dynamic, as evidenced by the progressive decline of certain clones and their replacement by genetically different lineages. Early strains of MRSA belonged to the sequence type (ST) 250, popularly known as the archaic clone which is a part of the highly successful clonal complex (CC) 8. This clone vanished from hospitals in European countries in the late 1970s and paved way for its descendants ST247 Iberian clone and ST239 Brazilian/Hungarian clone and subsequently for ST5 USA100 or ST36 EMRSA-16 which are genetically unrelated to CC8. The emergence, expansion and decline of MRSA clones is a poorly understood
phenomenon which is believed to be driven by many factors such as clone specific traits, selection pressure imposed by antibiotics and evolutionary process of this pathogen (Rasigade and Vandenesch, 2014).

Figure 1.1 Evolution of MRSA clones of CC8 is achieved by point mutations in housekeeping genes and the acquisition of different SCCmec types. The predicted ancestor of CC8 is ST8-MSSA. This diagram is drawn based on the evolutionary pattern described by Enright et al., 2002.

MRSA largely remained as a hospital-issue till the early 1990s when cases of MRSA infections were reported in community, affecting healthy and young individuals who had no traditional predisposing risk factors for MRSA infections (Udo et al., 1993). This newly ‘born’ lineage namely, community-associated MRSA (CA-MRSA) can be defined epidemiologically as MRSA isolated from outpatients with no history of hospitalization or invasive medical procedures in the previous year (Kallen et al., 2010). Clinical manifestations of CA-MRSA are usually pyogenic skin infections; however fatal diseases such as necrotizing pneumonia, necrotizing fasciitis have also been found to be associated with this pathogen. The strains of CA-MRSA differ significantly from their
hospital counterpart with respect to genetic background, antibiotic-susceptibility patterns, virulence and fitness determinants. CA-MRSA belongs to diverse clonal backgrounds such as ST1-IV (USA400), ST8-IV (USA300), ST80-IV (European clone), ST59-V (Taiwan clone), ST22-IV (EMRSA-15) etc. It usually harbors smaller SCCmec elements (type IV, V) and remains susceptible to non-beta-lactam antibiotics. The majority of CA-MRSA strains produce Panton-Valentine leukocidin (PVL), a phage-borne leukotoxin and it is often considered as a genetic marker of CA-MRSA. Though PVL has been proven to cause excessive tissue damage and inflammatory response, its role in pathogenesis, virulence and dissemination of CA-MRSA is still a matter of debate (Diep et al., 2008).

A single community-associated clone designated as USA300 based on its unique PFGE pattern (or ST8 by MLST) accounted for an unparalleled episode of CA-MRSA infections, especially in the United States by 2004 (King et al., 2006). USA300 have been implicated in skin and soft tissue infections (SSTIs) as well as in highly invasive infections (Limbago et al., 2009). This pandemic clone is endowed with many unique MGEs such as the S. aureus Pathogenicity Island 5 (SaPI5), ACME (Arginine Catabolic Mobile Element) gene cluster etc., which might have contributed partially to its virulence and the unprecedented success to persist in the community. SaPI5 codes for two enterotoxins, namely Seq and Sek, whereas the ACME and its associated speG gene help in the survival of this clone in the acidic environment of human skin (Joshi et al., 2011). Furthermore, USA300 is reported to over express the global transcriptional regulators agr and sae resulting in the increased expression of virulence genes (Li et al., 2010; Montgomery et al., 2010). USA300 was subsequently reported in other countries such as Pakistan, Japan, South Korea, Bangladesh and India (Shabir et al., 2010; Chuang and Huang, 2013; Paul et al; 2014; Rajan et al., 2015).

1.2 Coagulase negative staphylococci: ‘Accidental’ pathogens

Coagulase negative staphylococci (CoNS), especially S. epidermidis and S. haemolyticus which were previously thought to be harmless commensals have gained the status of pathogens in recent decades. It is often difficult for a clinician to assert whether a CoNS isolated from a sample is the real causative agent or an unspecific contaminant.
(Ziebuhr et al., 2006). *Staphylococcus epidermidis*, a benign colonizer of human mucosa is often referred as a pathogen that lives at the edge between commensalism and pathogenicity (Schoenfelder et al., 2010). Although not equipped with aggressive virulence factors such as a variety of toxins as seen in *S. aureus*, *S. epidermidis* has unique mechanisms that promote its persistence and asymptomatic transmission by evading human immune system. This involves protective exopolymers such as poly gamma glutamic acid (PGA) and polysaccharide intercellular adhesion (PIA), mechanisms to sense host-antimicrobial peptides, biofilm-forming ability etc. (Otto, 2008).

Biofilms consist of single or multiple species of bacteria attached to a solid surface and encased in an extracellular matrix composed of polysaccharides, proteins, extracellular DNA, wall teichoic acid etc. Biofilms differ markedly from planktonic cells on account of their reduced growth rate, increased drug resistance and greater adaptability (Halebeedu et al., 2014). Biofilm formation in *S. epidermidis* is mainly mediated by PIA, the synthesis of which is achieved by enzymes encoded by *icaADBC* operon (Heilmann et al., 1996). *Ica* genes are preferentially found in healthcare-associated clones of *S. epidermidis*. This species shows great diversity with 74 STs. The majority of the nosocomial isolates from different geographical areas were reported to be of ST2, a lineage which is positive for *ica* genes and the insertion sequence, IS256. This insertion element usually occurs in the genomes of staphylococci in multiple copies or as a part of the composite transposon Tn4001, which confers resistance to aminoglycosides (Hennig et al., 2008). Alternate insertion and excision of IS256 from *ica* operon have been shown to influence the gene expression and in turn causes phase variation of biofilm formation. IS256 also contributes significantly toward the genetic flexibility of this organism by mediating homologous recombination events (Schoenfelder et al., 2010).

*Staphylococcus haemolyticus* is the second most common CoNS implicated in blood stream infections (Takeuchi et al., 2005). It also causes urinary tract infections, endocarditis, peritonitis etc. It has emerged as an established nosocomial pathogen on account of its ability to acquire high level resistance to many higher end antibiotics such as glycopeptides and oxazolidinones (Falcone et al., 2006; de Almeida et al., 2012). The
peptidoglycan of *S. haemolyticus* is highly cross linked and contains serine instead of glycine in the cross bridge and this alteration interferes with the binding of glycopeptide (Klein et al., 1996). Whole genome sequence of the *S. haemolyticus* strain JCSC1435 revealed the presence of abundant insertion sequences (IS) which could be responsible for the extreme plasticity of this organism and aid in frequent genomic rearrangements, phenotypic diversification and acquisition of various drug resistance mechanisms (Takeuchi et al., 2005; Barros et al., 2012).

### 1.3 Epidemiological typing

Molecular characterization of pathogens plays an important role in the epidemiology of infectious diseases by generating adequate information for identification and tracking of outbreak strains to implement infection control strategies. Advancement in typing methodologies has revolutionized the field of molecular epidemiology. Typing enables the discrimination of epidemic clones involved in an outbreak by means of characteristics that differ from those of unrelated clones. An ideal typing method should be based on characteristics which are stable in the epidemic strain, but are adequately diverse within the species population (Witte et al., 2006). In case of *S. aureus*, serotyping and phage typing were the oldest phenotypic typing methods, which were later superseded by gel based typing such as pulsed field gel electrophoresis (PFGE) and sequence based analyses which include staphylococcal protein A (*spa*) typing, direct repeat unit (*dru*) typing, *ccrB* typing, multi-locus sequence typing (MLST) etc. Single locus sequence typing (SLST) methods such as *spa* and *dru* are based on VNTR (Variable Number Tandem Repeats) regions. MLST takes into account the variations in seven housekeeping genes and assigns a unique allelic profile known as a sequence type (ST) for each isolate. The ability to share sequence data through internet ([www.mlst.com](http://www.mlst.com)) has made MLST a tool for global epidemiology. MLST can be used for long term epidemiological surveillance as it indexes variations that accumulate slowly over time (Chambers and DeLeo, 2009), whereas PFGE remains the gold standard for outbreak detection on account of its ability to measure the differences spanning the entire genome (Singh et al., 2006) and higher discriminatory power. PFGE can detect a variety of genetic events which result in the rearrangement/insertion/deletion of restriction sites.
Despite its widespread acceptance, PFGE suffers from certain criticism such as its labor-intensity and poor inter-laboratory reproducibility.

More recently, whole genome sequencing (WGS) has proven to be a rapid and comprehensive epidemiological tool, as evidenced by three successful MRSA outbreak investigations in UK (Koser et al., 2012; Eyre et al., 2012; Harris et al., 2013). The WGS detects various genetic events such as variations in single nucleotide polymorphisms (SNPs), genomic rearrangements and/or movement of mobile genetic elements (MGEs), which are likely to occur during outbreaks. These information could be useful in clustering strains and thus to track MRSA outbreaks and reservoir identification in hospitals. The major obstacle to the implementation of WGS by clinical laboratories is the high cost of instrumentation and the management of huge amount of sequence data, the analysis of which requires bioinformatic expertise. In recent years, WGS has also been employed to trace the genetic ancestry of many successful MRSA clones such as ST22, ST30, ST8 and ST239 (Harris et al., 2010; DeLeo et al., 2011; MacAdam et al., 2012; Holden et al., 2013;Uhlemann et al., 2014a). Recent studies which analyzed a cluster of household USA300/ST8 strains from different cities in the US by WGS showed the endemicity of this clone in households which can serve as major long term reservoirs for the persistence, diversification and transmission of this pandemic clone (Uhlemann et al., 2014a; Alam et al., 2015). It has also been proposed that mutations in gyrA/grlA which are associated with fluroquinolone-resistance had a crucial role in the recent evolution of this clone (Alam et al., 2015).

1.4 The Indian scenario

Indian healthcare settings have been confronting with the menace of antimicrobial resistance, especially that of methicillin resistant staphylococci and ESBL-(extended spectrum beta-lactamase) producing Gram-negative pathogens. Tackling the issue of antimicrobial resistance is challenging in India on many grounds such as large population, over-the-counter (OTC) availability of antibiotics, lack of surveillance programmes, socio-economic disparity, sanitation issues etc. The problem of MRSA has now been well recognized in Indian hospitals as evidenced by a two-year nationwide study from 2008 by Indian network for surveillance of antimicrobial resistance (INSAR).
which reported an overall MRSA-prevalence of 41% in Indian hospitals (Joshi et al., 2013). The earliest reported clones of HA-MRSA from India belonged to ST239, the typical Hungarian/Brazilian clone which was gradually replaced by lineages such as ST22 and ST772 in Indian hospitals (Arakere et al., 2005; D’Souza et al., 2010; Shambat et al., 2012; Dhawan et al., 2014). This recent trend of CA-MRSA strains infiltrating hospital environment is a worrisome development because of the wider host range of CA-MRSA and the severity of the infections it causes.

We lag behind the medical fraternities of developed countries in areas such as national antibiotic stewardship, infection-control activities, concerted surveillance etc. Even though an antibiotic policy was proposed in 2011 in India, it has been put on hold as the recommendations were found to be difficult to implement. This has prompted different medical societies in India to organize a joint meeting to develop a ‘roadmap’ outlining the action plans to address the issue of drug-resistance (Ghafur et al, 2013). This initiative entitled, “Chennai declaration” has been widely acclaimed by various international medical bodies and policy makers (Holmes and Sharland, 2013; Ghafur, 2014). Rather than opting for a strict and perfect antibiotic policy, Chennai declaration has suggested a realistic and implementable policy which a country like India with no rationalization of antibiotic usage can easily adopt. The major recommendations include (a) the step-wise rationalization of OTC sale of antibiotics starting from higher-end ones and extending it to second- and first-line drugs, (b) setting up of hospital and regional infection control committees for active surveillance, (c) determination of molecular epidemiology of resistant strains (d) regulation of antibiotic usage in veterinary practice (e) promotion of public awareness programmes on antibiotic usage by print and electronic media etc.

Except for a few city-based studies which analyzed a limited number of strains, the molecular epidemiology of staphylococci from Indian subcontinent is largely unknown (Arakere et al., 2005; Nadig et al., 2006; D’Souza et al., 2010; Nadig et al., 2010; Nadig et al., 2012; Shambat et al., 2012). Our investigation was aimed at analyzing the population structure of drug-resistant staphylococci in the healthcare as well as community settings in the city of Mysuru, South India, which has not been explored
previously. By employing molecular epidemiological techniques we made an attempt to characterize a cluster of strains implicated in a variety of staphylococcal diseases in three major hospitals in Mysuru. The findings of our study cannot be extrapolated to other settings in the country. Nevertheless, our results give new insight into the emerging trends in the epidemiology of antibiotic resistant staphylococci in this geographical area. But a concerted and continuous nationwide effort is essential to study the changing epidemiology of staphylococci, not only to implement treatment and prevention strategies, but also to analyze the evolution of this pathogen.