Chapter III

Phenotypic determination of resistance
3.1 INTRODUCTION

The clinical categorization of microorganisms as resistant (R), intermediate (I) or susceptible (S) provides information on the likely outcome of treatment with the respective drug. In spite of many drawbacks, disc diffusion method remains the most preferred technique for resistance determination in routine clinical laboratories. The breakpoint of each antibiotic is set by analyzing MIC distribution, clinical dosage and pharmacokinetic/ pharmacodynamic (PK/PD) property of the drug. Various international organizations such as Clinical and Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST), British Society for Antimicrobial Chemotherapy (BSAC) etc. are authorized to publish and update the breakpoints for antimicrobial susceptibility testing.

A ‘susceptible’ isolate is the one which can be inhibited by the usually achievable concentration of the drug when the dosage recommended to treat the site of infection is used, whereas the category ‘intermediate’ implies an uncertain therapeutic effect when used at the recommended dosage of the drug, but assures a highly likelihood of clinical efficacy in body sites where the drugs are physiologically concentrated (e.g., β-lactams and quinolones in urine) or when a higher than the normal dosage of the drug can be used (CLSI, 2012). Interpretation as ‘resistant’ always implies a high likelihood of therapeutic failure when the recommended dosage of the drug is used.

As far as staphylococci are concerned, the most infamous resistance trait is the ‘methicillin-resistance’ which dates back to 1960s. It is a classical terminology that implies resistance to the entire class of β-lactam antibiotics, except for the recently introduced anti-MRSA cephalosporins such as ceftobiprole and ceftaroline (El Solh, 2009). An important feature of methicillin-resistance is that its expression can either be homogenous or heterogeneous. In the latter, the majority of the cells in the population remain susceptible under in vitro testing conditions and this makes the detection difficult. In order to obtain a better expression, CLSI has put forward many recommendations. This includes the use of cefoxitin disc (30 µg) as a surrogate for methicillin-resistance, incubation at a lower temperature (not exceeding 35°C), supplementation of the media with 2% NaCl and prolongation of the incubation time to 24 h etc. A strict adhererence to
these recommendations is important for the accurate detection of methicillin-resistance in *S. aureus* and coagulase negative staphylococci by employing disc diffusion method.

Treatment of hospital- associated MRSA (HA-MRSA) have been challenging as they are usually multidrug resistant, whereas community-associated MRSA (CA-MRSA) strains have traditionally been susceptible to non-beta-lactam drugs and can be treated with antibiotics such as clindamycin, gentamicin, trimethoprim-sulfamethoxazole, tetracycline etc. (DeLeo et al., 2010). But more recently, some note of caution has been expressed on the emergence of multidrug resistance among CA-MRSA strains (Diep et al., 2008; Wang et al., 2012). Vancomycin has been regarded for the past many decades as the mainstay of initial treatment for severe MRSA infections on account of its reasonably good safety profile and low induction of resistance, and also because of the unavailability of approved alternatives (Micek, 2007). But vancomycin suffers from many criticisms which include poor bactericidal activity, unachievable PK/PD targets, nephrotoxicity at high doses, poor penetration into lung fluids, a gradual increase in MIC among MRSA strains (MIC ‘creep’) etc. (Gould et al., 2011). Newer drugs such as linezolid, daptomycin and tigecycline have been shown to be effective as anti-MRSA drugs. Among them, linezolid is an entirely synthetic drug and the only oxazolidinone antibacterial approved for clinical use. Linezolid has 100% oral bioavailability and is associated with a lower risk of nephrotoxicity in comparison to vancomycin (Beibei et al., 2010). But long term usage of linezolid may have safety issues such as peripheral and optic neuropathies. There are other agents such as oritavancin, dalbavancin, delafloxacin and tidezolid which have shown promising results in clinical trials and would probably be approved by FDA in the upcoming years.

Although very rare in its occurrence, recent years have witnessed the emergence of vancomycin- and linezolid- resistance among staphylococcal strains, challenging two effective therapeutic options to treat infections caused by methicillin resistant strains (Thati et al., 2011; Gu et al., 2013). It is also important to note that these worrisome trends in resistance are more prevalent in coagulase negative staphylococci (CoNS) such as *S. haemolyticus* and *S. epidermidis*, reiterating the clinical importance of CoNS as major reservoirs of resistance genes. In the wake of emergence of resistance to high end
antibiotics, a strict surveillance is very important to prevent the transmission of resistant strains and also to preserve the clinical utility of these drugs.

3.2 MATERIAL AND METHODS

3.2.1 Study design and clinical isolates

This was a laboratory-based study involving the participation of three major tertiary care hospitals in the city of Mysuru, Karnataka state, South India during a period from April 2011 to December 2013. Among the three, two are teaching hospitals with bed strength of 1500, whereas the other one is with 500 beds. We analyzed a cluster of non-repetitive staphylococcal isolates (n=320) obtained as a part of routine clinical care in these hospitals. This included isolates from both outpatients and patients admitted to the hospitals. Our study was neither gender-biased nor restricted to any specific group of patients. Only the isolates with significant clinical implications as certified by the medical practitioners associated with our study were included for analysis. In case of multiple isolates from the same patient, only the first isolate was included.

Care has been taken in the case of coagulase negative staphylococci (CoNS) to exclude commensal isolates. Thus, cases of ‘true infections’ by CoNS were predicted based on the following criteria suggested by Beekman et al., (2005). This included (a) isolation of a strain in the form of pure culture from the infected site and repeated isolation of the same strain during the course of infection; (b) In the case of sepsis, two positive blood cultures within 5 days or one culture with clinical signs of blood stream infection. In the case of neonatal septicaemia, we adopted the definition proposed by Craft and Finer (2001) to define a true CoNS illness. They defined a true bacteremia as a positive blood culture of the same organism with >50 colony forming units (cfu/ml) from a peripheral site. Selection criteria of community-acquired MRSA (CA-MRSA) which were analyzed by various epidemiological tools in this study are described in chapter V of this thesis.

3.2.2 Phenotypic identification of the isolates

Preliminary identification of the isolates as staphylococci was based on standard microbiological observations. This included characteristic growth patterns on blood agar,
mannitol salt agar and DNase plates, Gram staining, catalase test, oxidase test and tube coagulase test using rabbit plasma (Himedia, India). The details of the preparation of media are given below (in g/l)

a) Blood agar base (Himedia, India): Beef heart peptone (10 g), tryptose (10 g), NaCl (5 g), agar (15 g), pH 7.3. Blood agar base (40 g) was suspended in 1 litre of distilled water and autoclaved. To the cooled media, 5% (v/v) defibrinated blood was added.

b) Mannitol salt agar: D-mannitol (10 g), NaCl (75 g), phenol red (0.025 g), peptone (10 g), beef extract (1 g), agar (15 g), pH 7.4

c) Toluidine blue DNA agar (Himedia, India): DNA (0.3 g), CaCl₂ (0.1 g), NaCl (10 g), toluindine blue (0.093 g), tris (hydroxymethyl) aminomethane (6.06 g), agar (15 g), pH 9

3.2.3 Identification of methicillin-resistance

All the isolates were screened for methicillin/oxacillin-resistance by growth on Mueller-Hinton agar plates (MHA) supplemented with 6µg/ml oxacillin (Himedia, India) and 2% NaCl. The plates were incubated at 35°C for 24 h. We also employed cefoxitin (30 µg) disc screening on MHA plates for detecting methicillin resistance. All the susceptibility testings were performed according to CLSI guidelines, 2012 (M100-S22).

3.2.4 Molecular identification of the species of methicillin resistant staphylococcal isolates

Species of methicillin resistant isolates were identified by multiplex PCR using species specific primers based on nuc genes as described by Hirotaki et al., (2011) or by employing 16S rDNA sequencing as per Lane et al., (1991).

Multiplex PCR was carried out in a volume of 50 µl with 200 µM each dNTP, 1X DreamTaq buffer (Thermoscientific, USA), 0.2 µM each primer, 2 µl of template DNA and 1.25 U DreamTaq DNA polymerase. The products were visualized by electrophoresing in 1X TAE buffer on a 1% agarose gel stained with ethidium bromide at a final concentration of 1 µg/ml. The details of the primers and PCR condition are given in table 3.1.
Identification of the species based on the sequencing of 16 S rDNA employed the universal primers 27F (5'-GAGTTTGATCCTGGCT-3') and 1492R (5'-TACGGCTACCTTGGTACGACTT-3'). PCR conditions involved an initial denaturation (94°C, 5 min); 35 cycles of denaturation (94°C, 15 s), annealing (56°C, 30 s) and elongation (72°C, 90 s); and a final elongation at 72°C for 7 min. The amplicons were sequenced and subjected to BLAST analysis.

**Table 3.1** Details of the primers used in multiplex PCR for species identification

<table>
<thead>
<tr>
<th>Species specificity</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>TCGCTTGCTATGATTGTGG</td>
<td>359</td>
<td>95°C 5 min</td>
</tr>
<tr>
<td></td>
<td>GCCAATGTTCTACCATAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>TTGTAAACCATTCTGGACCG</td>
<td>251</td>
<td>95°C 30 s</td>
</tr>
<tr>
<td></td>
<td>ATGCGTGAGATCTTCTTTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>TAGTGGTAGGCGATATTAGCC</td>
<td>434</td>
<td>58°C 30 s</td>
</tr>
<tr>
<td></td>
<td>ACGATATTTGCCCATTCCGTG</td>
<td></td>
<td>72°C 70 s</td>
</tr>
<tr>
<td>S. hominis</td>
<td>TACAGGGGCCATTTAAAGACG</td>
<td>177</td>
<td>72°C 2 min</td>
</tr>
<tr>
<td></td>
<td>GTTCTGCTGTATCAACACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>TTTGGATGCGATAGATTGG</td>
<td>843</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTTCAAGACTTTTCAAGGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**3.2.5 Antibiotic susceptibility test and MIC determination**

Susceptibilities of methicillin-resistant isolates to other antibiotics were determined by Kirby-Bauer agar disc diffusion method, following the guidelines of CLSI, 2012. Antibiotics included gentamicin (10 µg), erythromycin (15 µg), clindamycin (2 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), tetracycline (30 µg), quinupristin-dalfopristin (15 µg), teicoplanin (30 µg), rifampicin (5 µg) and linezolid (30 µg). Inducible resistance to clindamycin was checked by placing clindamycin and erythromycin discs 12 mm apart to analyze the formation D-zone (flattening of the zone towards erythromycin disc). All the isolates were classified as sensitive, intermediate or resistant according to the
interpretive criteria of CLSI M100-S22. Here we have not shown the data pertaining to susceptible or intermediate isolates. Methicillin resistant isolates which were resistant to at least one agent in three or more non beta-lactam antimicrobial categories were considered as multidrug resistant (Magiorakos et al., 2011). MICs were determined for selected isolates by using E-test strips (Biomerieux SA, France) on MHA plates incubated at 37°C for 24 h. For determining the MIC of oxacillin, the medium was supplemented with 2% NaCl and the plates were incubated at 35°C for 24 h.

3.3 RESULTS

3.3.1 Phenotypic identification of staphylococci

We categorized the isolates initially into coagulase positive or negative based on tube coagulase test. The former, in case of human clinical samples, is predominantly S. aureus. Species of the isolates were later confirmed using molecular approaches. All the isolates were Gram-positive, non motile, non-spore forming cocci, arranged in irregular clusters or in pairs or tetrads. All the coagulase positive isolates have shown distinct zones of beta-haemolysis around the colonies, whereas CoNS were either non-haemolytic, or haemlytic with clear or hazy zones. All the strains were catalase-positive and oxidase-negative. The majority (n=222, 69.3%) of our isolates were coagulase-positive and the remaining (n=98) were CoNS. Positive results for mannitol fermentation and DNase production further confirmed the identity of coagulase positive strains.

![Figure 3.1](image)

*Figure 3.1 a) Beta-haemolytic colonies on blood agar; b) Mannitol salt agar plate showing fermenting colonies in yellow and the non-fermenting isolates in pink; c) zone of hydrolysis by S. aureus on a DNase plate.*
3.3.2 Disease manifestations

Isolates analyzed in our study were implicated in various staphylococcal diseases ranging from common skin and soft tissue infections (SSTIs) to invasive illnesses such as bacteremia, neonatal sepsis, osteomyelitis, septic arthritis, surgical site infections etc. SSTIs included mainly impetigo, furuncle, carbuncle, cellulitis, abscesses of foot, breast and gluteal region, tissue necrosis etc. In the case of CoNS, the isolates were from SSTIs, bacteremia, neonatal sepsis and surgical site infections.

3.3.3 Detection of methicillin-resistance

Among the coagulase-positive staphylococcal isolates (presumably S. aureus), 86.9% (n=193) were found to be methicillin resistant and 80.6% (n=79) of CoNS were also resistant to methicillin as confirmed by the growth on oxacillin (6 µg/ml) containing MHA plates. The majority of the resistant isolates, both of S. aureus (68.4%) and CoNS (32.7%), were obtained from cases of skin and soft tissue infections (SSTIs) (fig 3.2).

![Screening of methicillin resistant isolates on an oxacillin plate; clinical manifestations of the methicillin resistant isolates of S. aureus and CoNS analyzed in the present study.](image)

**Figure 3.2** a) Screening of methicillin resistant isolates on an oxacillin plate; b) clinical manifestations of the methicillin resistant isolates of S. aureus and CoNS analyzed in the present study.

3.3.4 Molecular confirmation of the species

All the coagulase positive isolates have given positive amplicons for species specific nuc locus of S. aureus. Our collection of coagulase negative staphylococci were
only of two species, the majority being *S. haemolyticus* (n=67) and the remaining *S. epidermidis* (n=12). A few isolates which failed to give amplification for species specific loci were identified as *S. haemolyticus* by 16S rDNA sequencing. *S. aureus* COL and *S. epidermidis* RP62A were used as the positive controls in PCR screening. As we had no prototype strain of *S. haemolyticus*, an isolate confirmed to be of *S. haemolyticus* by 16S rDNA was used as the positive control.

**Figure 3.3** Amplification pattern obtained with multiplex PCR for species determination.  
*a*) Lane M: marker (250, 500, 750, 1000 bp, from the bottom); lanes 1, 2, 4-16: *S. haemolyticus* (SH); lane 3: *S. epidermidis* (SE).  
*b*) M-marker (100, 200, 300, 400, 500 bp, from the bottom); lanes 1-10: *S. aureus* (SA).

### 3.3.5 Antibiotic susceptibility test

Among the isolates of *S. aureus*, the highest resistance (86%) was recorded for ciprofloxacin, followed by erythromycin (51.8%) and quinupristin-dalfopristin (44%), whereas the resistance rates for chloramphenicol and tetracycline remained low (13% and 10.4% respectively). No resistance was recorded for the glycopeptide antibiotic teicoplanin and the oxazolidinone drug linezolid. A similar pattern of resistance was observed for coagulase negative staphylococci, with ciprofloxacin-resistance being the highest (69.6%) and with less isolates showing resistance to chloramphenicol and tetracycline. Species-wise resistance pattern is represented graphically (fig 3.6). None of the isolates of CoNS was resistant to teicoplanin, but notably five isolates of *S. haemolyticus* have shown resistance to the synthetic antibiotic, linezolid. All the linezolid-resistant isolates were also resistant to oxacillin, gentamicin, clindamycin and chloramphenicol. These isolates exhibited high MICs (>256 µg/ml) of linezolid, oxacillin, clindamycin and chloramphenicol (fig 3.4)
**Figure 3.4** a) Antibiotic susceptibility pattern of one of the linezolid resistant isolates (antibiotics to which the isolate was resistant are labeled); b & c) MICs of linezolid and clindamycin (>256 µg/ml).

**Table 3.2** Clinical details and resistance profile of linezolid resistant isolates of *S. haemolyticus*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Hospital</th>
<th>Clinical manifestation</th>
<th>Antibiotic resistance profile</th>
<th>Previous exposure to linezolid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>cellulitis</td>
<td>GEN, CLI, CHL, RIF, SXT</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>bacteremia</td>
<td>GEN, CLI, CHL, ERY, CIP, Q-D</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>surgical site infection</td>
<td>GEN, CLI, CHL, ERY, CIP</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>orthopedic infection</td>
<td>GEN, CLI, CHL, ERY, CIP</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>surgical site infection</td>
<td>GEN, CLI, CHL, ERY</td>
<td>No</td>
</tr>
</tbody>
</table>

We found it interesting to note relatively high incidences of neonatal septicaemia caused by *S. haemolyticus*. They were ruled out to be contaminants based on various criteria which have been discussed. Among these isolates, 78.2% (n=18) were multidrug resistant, with the majority of the strains exhibiting resistance to erythromycin and quinupristin-dalfopristin. Inducible resistance to clindamycin was observed in 30.6% (n=59) isolates of *S. aureus* and it was 25.5% (n=25) among CoNS (fig 3.5 a). One of the linezolid resistant isolates exhibited an uncommon resistance pattern of erythromycin-sensitivity & clindamycin-resistance (fig 3.5 b). Multidrug resistance (resistance to ≥3 classes of non-beta-lactam drugs) was observed in 62.2% (n=120) of the methicillin resistant *S. aureus* isolates, whereas it was found to be 72% (n=57) in the case of CoNS.
**Figure 3.5** a) A plate showing positive D-zone test, which indicates inducible resistance to clindamycin. Erythromycin (a macrolide) induces resistance to clindamycin (a lincosamide), as evidenced by the flattening of zone the appearance of resistant colonies; b) One of the linezolid resistant strains exhibiting a rare phenotype of erythromycin-susceptibility & clindamycin-resistance (M$^S$/L$^R$).

**Figure 3.6** Resistance profile of methicillin resistant (MR) isolates towards other antibiotics

*abbreviations of antibiotics: GEN gentamicin, ERY erythromycin, CLI clindamycin, CIP ciprofloxacin, SXT trimethoprim-sulfamethoxazole, CHL chloramphenicol, TET tetracycline, Q-D quinupristin-dalfopristin, RIF rifampicin, TEI teicoplanin and LZ linezolid.
3.4 DISCUSSION

Antimicrobial resistance has been a compelling subject in the area of infection-control and prevention. Among Gram-positive bacterial pathogens, staphylococci are historically known for their extraordinary ability to keep themselves one step ahead of our therapeutic efforts. Both *S. aureus* and coagulase negative staphylococci now share the equal status of clinical pathogens, in contrast to the olden days when clinical laboratories used to discard coagulase negative staphylococcal isolates encountered in clinical samples as contaminants, without assessing their role in infection. While *S. aureus* causes a variety of skin infections and toxin-mediated diseases, species of CoNS are usually confined to device-associated and blood stream infections. In regard to drug resistance, both the groups have shown loss of susceptibility to many antibiotics during the course of their evolution. Drug resistant CoNS pose, besides their potential as pathogens in their own right, an additional risk by serving as reservoirs for the transmission of resistance genes to other, more pathogenic bacteria.

This chapter of the thesis describes the initial screening of a collection of clinical isolates of staphylococci for antibiotic resistance. The reliable identification of the pathogen is the first and foremost challenge in antimicrobial chemotherapy, especially in resource poor settings. Here we employed a combination of three tests for the confirmation of *S. aureus*. This included tube coagulase test using rabbit plasma and growth on mannitol salt agar as well as deoxyribonuclease (DNase) plates. A positive result for all the three tests confirmed the pathogen as *S. aureus*. A negative result for tube coagulase test was corroborated by negative results for the remaining two tests. The efficiency and sensitivity in identifying *S. aureus* by sequel testing of the culture in this way has been validated in a study by Katatee et al., (2010). We also confirmed the identity of the species by molecular approach and these results were in cent per cent agreement with the preliminary categorization based on the above tests. The majority (71%, n=193) of our collection of methicillin resistant isolates were *S. aureus* (MRSA) and among those of coagulase negative staphylococci we observed a high prevalence of *S. haemolyticus* (84.8%, n=67). We encountered only a few *S. epidermidis* (n=12) isolates, but all of them were methicillin resistant and were implicated in cases of bacteremia or surgical site infections.
MRSA continues to be a major burden in Indian healthcare settings. Literatures are available on quite a few hospital based studies from India reporting high prevalence of MRSA. Anupurba et al., (2007) reported 54.8% of *S. aureus* as MRSA, with 80% of them being multidrug resistant, in a hospital located in Uttar Pradesh, North India. A nation-wide survey by the Indian Network for Surveillance of Antimicrobial Resistance (INSAR) in 2008 and 2009 reported an overall MRSA prevalence of 41% in Indian hospitals (Joshi et al., 2013). A recent study by Bouchiat et al., (2015) in a Bangalore-based hospital reported an MRSA rate of 52.2% with resistance rates of 70.6% and 54.3% to ciprofloxacin and erythromycin respectively. In our study we observed a high (86.9%) rate of MRSA in a limited sample size of 223 *S. aureus*. Resistance to ciprofloxacin and/or erythromycin was conspicuous among our isolates. We also observed inducible clindamycin resistance in 30.6% of the MRSA isolates. Detection of this pattern of resistance has immense clinical importance, especially in cases of CA-MRSA, as clindamycin is one of recommended drugs to treat CA-MRSA infections. None of our isolates were resistant to the glycopeptide antibiotic teicoplanin. Vancomycin (glycopeptide) resistance in staphylococci is still considered rare; however, reports on reduced susceptibility or resistance towards vancomycin have been reported from India (Banerjee and Anupurba, 2011; Thati et al., 2011; Tiwari and Sen, 2006).

Studies from India also reveal a high rate of nasal carriage of MRSA. In a community-based screening for MRSA revealed that 18.1% of healthy parents attending a baby clinic in Delhi were carriers (Saxena et al., 2003). Another study among children of age below five years in Ujjain reported a prevalence rate of 6.3% for *S. aureus* and 16.3% of them were MRSA (Pathak et al., 2010). An extension of this work which screened 1002 children attending preschools in Ujjain reported 35% nasal carriage of *S. aureus* and 29% of MRSA (Dey et al., 2013). This high rate of nasal carriage of MRSA is a disturbing trend. A recent study by Govindan et al., (2015) which analyzed the nasal carriage of *S. aureus* in 1503 school children of the age group 5-16 years in Udupi taluk, Karnataka state reported the prevalence rate as 29.3%. But the MRSA carriage was found to be low (1.1%) among these children and all the isolates of MRSA from this study was found to be susceptible to non-beta-lactam drugs.
Besides MRSA, we observed quite a few number of methicillin resistant CoNS which accounted substantially for blood stream infections and also for SSTIs. Among our isolates of CoNS, the important resistance trait to mention is the linezolid-resistance observed in five isolates of *S. haemolyticus*. Linezolid is a synthetic drug licenced to treat severe infections caused by multidrug resistant strains of staphylococci, enterococci and other Gram-positive pathogens. The low occurrence of linezolid-resistance is mainly attributed to the absolute synthetic nature of this antibiotic for which natural mechanisms of resistance are not much envisaged. Among the isolates, three were from one hospital, obtained within a period of two months and the remaining two, from another hospital were obtained nearly five months in gap. The first isolate was from a 60 year old male who was admitted the hospital following edema and cellulitis of left lower limb. He presented with an open sore and redness in the infected area with pus filled bumps. The patient had been undergoing dialysis in another hospital for the past six months. He had an exposure to linezolid (600 mg twice daily for a period of 28 days) for the treatment of MRSA bacteremia which he developed during the course of dialysis. The remaining isolates were from bacteremia or surgical site infections and the patients had not undergone linezolid therapy previously. High MIC values (>256 µg/ml) of linezolid, oxacillin, clindamycin and chloramphenicol were observed for all the isolates. Linezolid resistance in *S. haemolyticus* is considered rare; however there have been reports from China, Brazil, Italy, Spain (Cui et al., 2013; de Almeida et al., 2012; Mazzariol et al., 2012; Rodriguez-Aranda et al., 2009). Literature from India documents a few cases of linezolid resistance in *S. haemolyticus*, other cogulase negative staphylococci and also in *Enterococcus faecalis* (Kalawat et al., 2011; Peer et al., 2011; Gupta et al., 2012; Rai et al., 2015).

In our surveillance, we noted incidences of methicillin resistant *S. haemolyticus* (n=23) in causing neonatal septicaemia in the NICU (neonatal intensive care unit) of one of the hospitals. Septicaemia is known to be a major cause of morbidity and mortality among newborns (Hamer et al., 2015). It is categorized into two types: early onset septicaemia which occurs within the first 72 h of life and late onset septicaemia which presents anytime between 72 h to 90 days of life. A clear distinction between a pathogen and a contaminant of CoNS in case of bacteremia is often difficult and we lack a clinical
In case of neonatal septicaemia, there have been reports on potential clinical impacts of CoNS on neurodevelopment (Nash et al., 2013). We considered our isolates as non-contaminants as the infants gave repeated positive blood cultures of *S. haemolyticus* in cfu >50. Out of the 23 cases, 18 were early onset-septicaemia and were associated mainly with two high risk factors: very low birth weight (VLBW, < 2500 g) and perinatal asphyxia. In the late-onset infections (n=5), 3 infants had undergone invasive medical procedures, whereas the other two were preterm babies. Apart from *S. haemolyticus* which formed the majority of the isolates implicated in neonatal septicaemia, *S. aureus* was implicated in 5 cases of early-onset septicaemia in infants with VLBW. Among them, two had the complications of intrapartam maternal fever. A 9-month study which analyzed 660 cases of late-onset septicaemia at the NICU of a teaching hospital in Lucknow, North India reported *S. haemolyticus* (34%) as the commonest causative agent among the various CoNS species (Jain et al., 2004). Recent studies from India reported *Klebsiella pneumoniae* and *Staphylococcus aureus* as major pathogens implicated in septicaemia in newborns (Kamath et al., 2010; Rastogi et al., 2010). Moreover, extended spectrum beta-lactamase (ESBL) and New Delhi metallo-beta-lactamase (NDM)- producing strains of *K. pneumoniae* have been reported to cause additional burden to treatment strategies (Rastogi et al., 2010; Datta et al., 2014). Jain et al., (2011) reported increased incidences of intravenous device associated bacteremia in the paediatric ward of a tertiary care hospital in North India due to followed by *S. haemolyticus*. *S. epidermidis*

In summary, we report a very high incidence of methicillin resistance (85%) in a convenience sample of 320 staphylococcal isolates collected from three hospitals in Mysore. Another important aspect of our observation is the clinical significance of the isolates of coagulase negative staphylococci identified in the study. It is noteworthy, particularly in the healthcare as well as research settings in India, where less attention is paid towards ‘non-aureus’ staphylococci. The aspect of CoNS as reservoirs for staphylococcal virulence traits with the potential to spread, via horizontal gene transfer, to *S. aureus* is gaining much importance in the recent years. Here, we also document the emergence of linezolid resistant strains of *S. haemolyticus* in this geographical region. This is indeed a cause of concern as linezolid has been a promising anti-MRSA drug
comparable in its spectrum of activity to that of vancomycin. Our study sheds light on the need for strict surveillance of linezolid resistant strains in hospitals in India. Moreover, to effectively prevent the transmission of linezolid-resistance, this drug should be treated as ‘reserve’ by clinicians.