CHAPTER-5

DISCUSSION

Discussion

In this study, 444 *S. aureus* were isolated from clinical as well as CA/environmental samples in order relate the frequency of virulence factors like biofilm production, exotoxin production and enterotoxin production.

The isolation of *S. aureus* from clinical as well as CA/environmental samples reflects it's ubiquitous occurrence as previously reported (Moore and Lindsay, 2001). The isolation rate of 50% and 52% in clinical and CA/environmental isolates respectively is in agreement with that obtained in earlier studies from clinical and environmental specimens (Arciola *et al.,* 2002). The occurrence of the pathogen in environmental samples (52%) may indicate poor infection control and unhygienic practices in the environment, which could serve as a reservoir of the organism.

The results revealed the total prevalence of *S. aureus* in both clinical and environmental samples, corresponding to 50.91% of all samples isolated from South Assam region of India. It was found that the prevalence of clinical *S. aureus* isolates were half of the total clinical samples (50.09%) collected for the study. The highest numbers of *S. aureus* were obtained from pus, followed by sputum and urine. This finding is exactly in conformity with those reported from Nigeria (Fayomi *et al.,* 2011). Also, majority of reports of *S. aureus* prevalence in India were reported from pus samples (Dar *et al.,* 2006; Nadig *et al.,* 2006).

Prevalence of *S. aureus* and sex differentiation were 47.4% and 54.4% in males and females respectively. Similar finding was reported by Shakya *et al.,* (2010) and contrast finding was revealed by Ahmad, (2010) (males- 42%, females- 57% and males-22.5%, females- 44.6% respectively).

Environmental/CA associated *S. aureus* revealed its presence in 52.17% among all the environmental samples collected. Normal skin flora showed maximum prevalence of *S. aureus* colonization (80%) among the environmental/ CA associated samples collected. Thus

S. aureus colonization in environmental/CA associated samples were slightly more prevalent than clinical samples. This finding is in accordance with the result obtained in the study conducted by Joshi *et al.,* (2013). The frequency of *S. aureus* colonization on different clinical and environmental settings might be due to the fact that this microorganism possesses certain mechanisms that favour its adaptation to some sites, with this species being the most prevalent bacterium in human skin and mucosa (Gemmell, 1986).

All the *S. aureus* isolates were sensitive to vancomycin (100%) and linezolid (100%). This was statistically significant ($p < 0.05$) compared with the other drugs used in the study. This is in agreement with previous studies (Kesah *et al.,* 2003; Shittu and Lin, 2006). Penicillin and ampicillin showed the highest resistance of 100% in clinical isolates. Whereas, environmental isolates showed a high resistance against ampicillin (100%) and penicillin (96%). This study was in accordance with the work of Rao *et al.,* (2011) who reported *S. aureus* isolates having 100% resistance with penicillin and 100% sensitive with vancomycin. However the result of present study contradicts *w.r.t.* linzoid resistance with earlier reports (Rao *et al.,* 2011; Venkatesh *et al.,* 2014) where 5.5% and 2.72% linzoid resistant isolate were reported respectively. It may be speculated that the effectiveness observed with the drug might be due to its high cost in our environment making it less readily available and hence less misused.

Methicillin resistance was in environmental isolates were slightly higher (25%) than clinical isolates (23%) among the *S. aureus* isolates, by the disc diffusion test. This result is in accordance with the study carried out by Rongpharpi *et al.,* (2013). Among total isolates, 24% isolates were found resistant to methicillin in this study that correlates to 34.2% as reported by Diekema *et al.,* (2004). However Prashini *et al.,* (2004) reported methicillin resistance by 56.25% of the isolates tested. The result of our study is in disagreement with the

work of Bhavsar *et al.,* (2015) who reported prevalence of MRSA was 43.33% and INSAR (Indian Network for Surveillance of Antimicrobial Resistance) group, India, which shows prevalence of MRSA was 41% in the year of 2008-2009 (INSAR, 2013). In our study none of the MRSA isolates found sensitive to ampicillin. Similar data was reported by Vidhani *et al.,* (2001). In this study the Prevalence of total MRSA was found to be almost 24% which is comparable to the study by Pai *et al.,* (2010), 29.1% and low in comparison to Saikia *et al.,* (2009) and Joshi *et al.,* (2013) who reported prevalence of MRSA, 34.7% and 41% respectively. This variation might be due to several factors like efficacy of infection control practices, healthcare facilities and antibiotic usage. Higher percentage of resistance to many antibiotics in the present study could be due to the illogical antibiotic usage and easy availability at the drug store without prescription.

Ciprofloxacin resistance in our study in clinical isolates was 23% and 34% in CA/environmental isolates. This result were similar to the result reported by Majumder *et al.,* (2001) who observed 22.8% resistance against ciprofloxacin but contradicts with the study of Rajaduraipandi *et al.,* (2004) and Maple *et al.,* (1989) who reported 12.8% and 17% resistance which is low in comparison to our result and Pulimood *et al.,* (1996) showed 90% resistance to ciprofloxacin which is again much higher as compare to our results.

Resistance to gentamycin was higher in environmental isolates (23%) than clinical isolates (21%). This results was supported by the work of Rajaduraipandi *et al.,* (2004) who reported 20.5% gentamicin resistant isolates. In another work done by Majumder *et al.,* (2001) and Pulimood *et al.,* (1996) revealed 94.1% and 85.5% gentamycin resistance which is much higher as compared to our results.

Higher percentage of Intermediate sensitivity was noted against antimicrobial agents like erythromycin in clinical isolates (35%) and (36%) in environmental isolates. Amikacin showed similar intermediate sensitivity pattern (30%) in both clinical and environmental

isolates. However, in a study by Rajaduraipandi *et al.,* (2006) higher percentage of intermediate sensitivity was noted against erythromycin, ofloxacin, amikacin, ciprofloxacin and cephotaxime. Although the majority of these multidrug- resistant isolates originated from clinical samples, isolates of environmental origin also showed a little less or almost same pattren. Pellegrino *et al.,* (2002) also reported that the use of antibiotics in hospital and the community at large serves as a major selective pressure for antibiotic resistant bacteria. Therefore it may be concluded that the same situation might have been responsible for the multidrug - resistance pattern observed in the present study.

In this study, evaluation of biofilm formation was studied by the CRA plate test, which is easier to perform and less time-consuming than staining methods. This method has been used successfully for the detection of biofilm-forming strains of *S. epidermidis* (Handke *et al.,* 2004; Oliveira *et al.,* 2006). However, the interpretation of results has been divisive as the phenotypic colony changes took place during incubation and thus false negative results had been observed. Moreover, it was very difficult to classify the colonies as biofilm producers or non-producers by mere observing the borderline colour of the colonies. Similar findings were reported by many authors (Vasudevan *et al.,* 2003; Milanov *et al.,* 2010).

In this study, CRA method showed lower sensitivity and specificity as compared to TM of biofilm evaluation. This methodological problem was also addressed by Mathur *et al.* (2006) who compared CRA, TCP and TM phenotypic methods used for determination of biofilm formation. Their results showed that the TM is the most sensitive method for biofilm formation analysis over CRA method. The low effectiveness of the CRA method in evaluation of biofilm production, was also shown by de Silva *et al.* (2002). Thus the unreliability of the CRA method was earlier presented by other authors (Atshan and Shamsudin, 2011; Rohde *et al.,* 2001, Vancraeynest *et al.,* 2004) and now confirmed in this

study. According to Arciola et al. (2002), CRA test results were correlated to the presence of *icaA* and *icaD* genes (Vázquez-Sánchez *et al.,* 2013), which are usually involved in staphylococcal biofilm formation. However, in this study we have observed a lack of correspondence between biofilm production by CRA method and presence of *ica* genes. This report is also supported by Ciftci *et al.,* (2009) and Zmantar *et al.,* (2010). Therefore, though CRA method is a convenient method for detection of *in-vitro* biofilm production, it cannot be correlated with molecular method for presence or absence of *ica* genes.

Superior results for biofilm formation were obtained using the quantitative phenotypic test (TM). The TM revealed that 76 (57.6%) of strains produced biofilm, while remaining 56 strains showed negative response in this test. All of 132 strains tested were shown to harbour the *icaA* and *icaD* genes, what makes them potential biofilm producers. The results are in accordance with work done by Vasudevan *et al.,* (2003) who detected the presence of both genes in 36 strains of *S. aureus* isolated from milk of cows suffering from mastitis. However, studies by Dhanawade *et al.,* (2010) reported that only 35 strains were positive for *S. aureus* among the group of 102 mastitis isolates from India, which is not in accordance to the present study.

Previous work has highlighted an increase in biofi1m formation in response to increased NaCl concentration (Lim *et al.,* 2004) which was also confirmed by the results of this study. An NaCl conc. of 0.75% was found to be statistically significant in inducing biofilm formation in both clinical and environmental isolates while high concentration of NaCl inhibits *in-vitro* biofilm formation. Some reports have proven that the cells within a biofilm are under greater osmotic stress than planktonic cells, and also that high osmolarity potentially inhibits biofilm establishment (Rinaudi *et al.,* 2006).

Biofilm formation on polystyrene and glass surfaces was comparatively decreased than PVC material by both clinical and CA/environmental isolates. This may be due to an

increased negative charge at the cell surface, suggesting an electrostatic repulsion (Neuhaus and Baddiley, 2003) in glass and polystyrene surfaces.

In our present study, pH of the cultured medium and incubation period were found to influence biofilm formation. At both acidic and alkaline pH the majority of low-grade biofilm production (OD_{620} ≤0.220) were observed which was also confirmed by Zmantar *et al.,* (2010). Our results are in agreement with Hamadi et al. (2005) who found cells adhere to glass weakly at highly acidic and alkaline pH levels. Previous studies have described the impact of acidic pH on biofilm. While the drop of pH value ≤4.0 strongly inhibited biofilm (Zmantar *et al.,* 2010 ; Tang *et al.,* 2012), pH values of 5.0–6.0 determined a slight decrease in the biofilm formation (Mafu *et al.,* 2011). The results ind*ica*ted that the maintenance of intracellular pH homeostasis is the basis of the enhanced physiological status and acid tolerance of biofilm cells.

Our experiment revealed that all of the MRSA isolates attained maximum biofilm density after exposure to sublethal doses of oxacillin. MSSA isolates, on the other hand, were biofilm producers advertently without exposure to any stress. Similar findings were confirmed by Mirani *et al.,* 2015 who suggested that, this might be due to oxacillin as it seems to be a stress factor responsible for induction of biofilm formation in MRSA isolates.

Our study revealed that the adherence capacity of SCV to glass materials (OD_{620nm}=0.82±0.40, mean±SD) was significantly (P<0.05) higher than that of the WT isolates on the same material (OD_{620nm}=0.55±0.28, mean±SD), which is in agreement with the reports regarding significant relationship (P<0.05) between biofilm formation in WT and SCV isolates (Singh *et al.,* 2009).

In vitro quantifi*ca*tion of biofilm formation in two distinct roots (clinical and CA/environmental) of *S. aureus* was performed to investigate the differences to established biofilms. The present study showed significant increase in biofilm production by both clinical

and CA/environmental isolates of *S. aureus* when exposed to increasing concentration of glucose and NaCl. This study revealed that at 1% glucose, enhanced biofilm formation of *S. aureus* was strongly associated with both clinical and CA/environmental isolates and observed in 85% of these isolates. Similar results were reported by Moretro *et al.,* 2003; Jahannes *et al.,* (2002) and Seidl *et al.,* 2008; whose work revealed that supplementation of 0.2 % glucose in TSB is sufficient to induce a visible biofilm formation and a further increase in glucose concentration up to 1% increases the biofilm formation significantly. This suggests that biofilm formation in *S. aureus* may be strongly dependent on addition of sugar. This finding was supported by the work of Mathur *et al.,* (2006) which specified that the use of sugar supplementations is essential for biofilm formation. However, this result contradicts with the work done by Croes *et al.,* (2009) who reported enhanced biofilm formation at 0.1% glucose strongly associated with MLST CC8 and observed in 60% of these isolates. Other authors also reported that biofilm formation was induced by increasing glucose concentrations up to 0.5% in both MRSA and MSSA isolates (O'Neill *et al.,* 2007; Rode *et al.,* 2007) which is again in contradiction with our results.

In the present study, biofilms treated with sub-lethal doses of oxacillin showed clusters of live (green) under CLSM. Despite the fact that biofilms exposed to oxacillin had smaller amounts of biofilm biomass, the typical high density cell clusters were detected. SEM analysis also revealed the same result with CLSM and detected enhanced biofilm matrix of the tested isolate under oxacillin exposure as compared to control.

In our study, we examined the ability of biofilm production in *S. aureus* by detecting *icaABCD* genes in all isolates. Of the 444 isolates (59%) isolates were positive for *icaA* gene and 47% were positive for *icaD* gene but no isolate was positive for *icaB* and *icaC* gene. Cifti *et al.,* (2009) examined the group of 59 isolates from mastitis and found only 16 *icaA* positive stains, 38 strains harboured the *icaD* gene and 15 of them contained both genes. On the other

hand, several authors showed presence of the *ica* locus genes in all *S. aureus* clinical isolates analyzed in their studies (Fowler *et al.,* 2001; Rohde *et al.,* 2001; Knobloch *et al.,* 2002; Asthan and Shamsudin, 2011 and Szweda *et al.,* 2012). These variations could be due to circulations of different clones of *S. aureus* in different regions. Presence of *icaA* or *icaD* negative biofilm positive isolates can be accounted for by an *ica* gene independent control of slime production/adhesion mechanism (Liberto *et al.,* 2009). On the contrary, inability of *Staphylococcus* isolates that were positive for *icaA* and/or *icaD* genes to produce biofilm *in vitro* can be due to point mutation in the locus and/or any other yet unidentified factors that negatively regulate polysaccharide intercellular adhesion synthesis or influence biofilm formation (Cramton *et al.,* 1991). Qin *et al.,* (2007). The authors showed that the *aap* and *bhp* genes may be involved in an alternative PIA-independent mechanism of biofilm formation, thus indicating that the absence of *icaABCD* operon genes does not exclude biofilm formation. On the other hand, inability of biofilm formation in some staphylococcal strains, despite the presence of *ica* genes can be caused by insertion of a 1332-bp insertion element (IS256), in *icaA* gene and causing its inactivation (Kiedrowski and Horswill, 2011).

In this study a low prevalence of *bap* genes (3.15%) were detected among all *S. aureus* isolates. These results are in agreement with the previous reports on *S. aureus* by Cucarella et al. (2001) who found only 5% isolates positive for *bap* gene. Szweda et al. (2012) did not report *bap* gene in any of isolates in his study in Poland. However, *bap* gene in clinical isolates was low as 1.1% which confirms the hypothesis proposed by Vantor *et al.,* (2008), that the *bap* gene has not spread among the *S. aureus* isolates of animal and human origin and its prevalence is very low.

In our study it was established that 70% isolates were positive for haemolysis production whereas 30% isolates did not show any type of haemolysis pattern. This result is

in contradiction with the studies by Younis *et al.,* (2000) who reported that out of 400 *S. aureus* isolates, 62.7% were found to be non-haemolytic. The results of our study also showed that the frequency of *hla* + *hlb* genes concurrently was 16.7% among all the isolates. Interestingly, in separate gene frequency analysis, each of *hla* or *hlb* genes alone were revealed to be the most frequent ones. Although both *hla* and *hlb* genes were harboured by the isolates, *hlb* gene was significantly higher in our study. Similar results were revealed by the studies of da Silva *et al.,* (2005) and Gharsa *et al.,* (2012). However our result disagrees with Alfatemi *et al.,* 2014 who showed coexistence of *hla*+*hld* gene in 48.83% isolates. The existence of *hla* and *hlb* genes in *S. aureus* isolates are important for this strains, related to staphylococcal infection cases that caused food poisoning.

In our study, the production of enterotoxins (*sea, seb, sec* and *sed*) were tested in all of 444 coagulase-positive *S. aureus* isolates and it was found that *sea* gene was prevalent in 65% of the isolates while *sed* gene was present in 45% of the isolates, which is in accordance to the study performed by Peck *et al.,* (2009) in Korea. None of the isolate carried *sec* and *sed* genes in our study. This result was supported by the studies of Bautista et al. (1988) who detected highest production of *sea* and *sed* isolated from sheep milk. The findings of the study is consistent with the study by Sina *et al.,* 2013 who reported absence of *sec* gene in all the isolates but contradicts with the point that prevalence of *seb* gene (44%) is higher than *sea* gene (32%). Similarly, Malaysian *S. aureus* strains had been reported to not harbour these genes (Lim *et al.,* 2012).

Kateete *et al.,* (2011) showed the prevalence of *sea* gene 85% among clinical isolates. In a study performed in the United States, the frequency of the *sea* gene was reported in the range of 54-95% (Shukla *et al.,* 2010) which is much consistent with result. The results partly explained the fact that samples were contaminated in nature. Study performed by Alfatemi *et*

al., (2014) reported the prevalence of *sea* gene was 27.39% which is little contradictory with our result. The frequency of the *sed* gene in our study was 45%. Our results contradicts with those of previous studies done by Shukla *et al.,* 2010, in a study from the United States, where prevalence of *sed* gene was reported to be in the range of 0-13%. The results of this study are also in agreement with the data reported from other countries (Scherrer *et al.,* 2004; Loncarevic *et al.,* 2005; Akineden *et al.,* 2008; Rall *et al.,* 2008; Pourmand *et al.,* 2009). The predominance of enterotoxins A and D contradicts reports from countries such as Brazil, Norway (da Silva *et al.,* 2005; Jørgensen *et al.,* 2005), where *sec* producing *S. aureus* were frequently isolated from milk and raw milk cheeses. However, Normanno *et al.,* (2007) showed that in Italian dairy products most of the isolated strains produced *sed,* followed by *sea, sec, and seb.*

In this study 94% isolates were positive for TNase production while 6% isolates did not produce TNase. However, production of this enzyme is a characteristic feature of *S. aureus* strains and sometimes this test is considered as 'Gold standard' for biochem*ica*l identif*ica*tion of *S. aureus*. Similar results were obtained by Benett *et al.,* (1986) who reported that 93% strains were TNase positive, isolated from foods and food ingredients. However, Chang and Huang (1995) reported that 99.5% of the strains produced this enzyme.