CHAPTER-3

MATERIALS AND METHODS

3.1. Study area:

The present study was conducted in south Assam part of India, popularly known as Barak Valley, covering three districts viz,

- Cachar (24° 22' to 25° 8' N Latitude and 92° 24' E to 93° 15' E Longitude) having a geographical area of 3786 sq. km and population density of 17,36,319 as per census, 2011. (source: cachar.gov.in).
- Karimganj (24°15' and 25°55' N latitude and 92°15' and 92°35' E longitudes) covering a geographical area of 1809 sq. km having a population density of 12,17002. (source: karimganj.gov.in).
- Hailakandi (24°68"33' N Latitude and 92°56"67' E Longitude) covering a geographical area of 1327 sq. km and populated by 65,9296 publics as per census 2011. (source: hailakandi.nic.in)

Geographically, the study area is surrounded by North Cachar Hills and Jaintia Hills in north, in east by Manipur, in south by Mizoram and in west by Tripua state. The topography of the area varies from small hillocks to plain areas and low lying areas.

3.2. Bacterial isolates:

3.2.1. Sample collection:

A total of 977 nos. non-repetitive clinical and environmental isolates were collected between March 2012 to February 2013 for the experimental study. Clinical samples were collected from common collection centre of Silchar Medical College and Hospital (SMCH) for the three districts (Cachar, Karimganj and Hailakandi) covering study area. The samples of clinical origin were collected from pus, sputum, throat swab, oral swab, nasal swab and

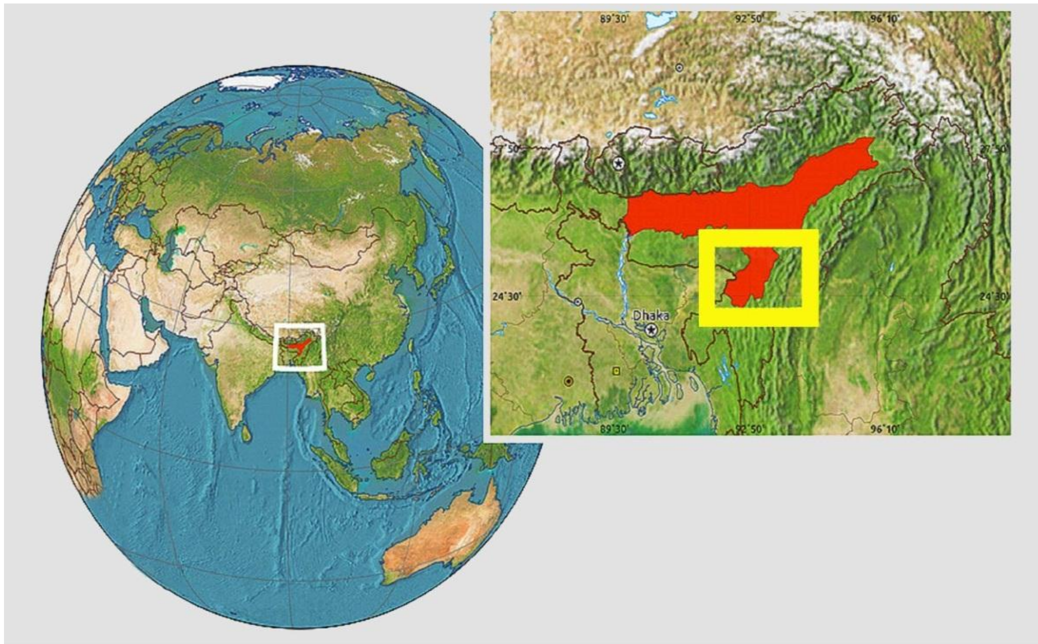

Figure 3.1: Map showing global location of south Assam.

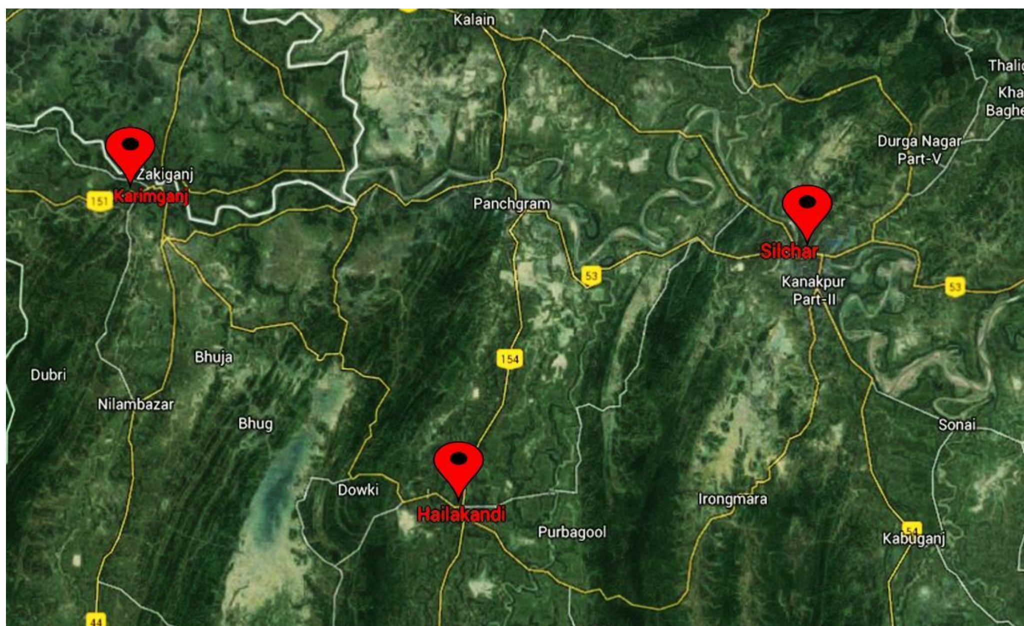

Figure 3.2: Map of the study area showing location of three districts of south Assam (Cachar, Karimganj and Hailakandi).

urine. All the samples were collected by standard procedure using sterile cotton swabs (BBL culture swab, BD diagnostics) except sputum and urine. Various demographic factors (age, sex, locality and occupation) and geographical factors (rural, urban, semi-urban, and semi-rural) were also recorded as baseline data.

Environmental samples included milk (both raw and packaged), skin flora of milk-handlers, surface of dairy equipments, bacteria of meat surface and its surroundings and normal skin flora (swab of anterior nares, perianum, arm and armpit). Food samples included readymade sweet (rasgulla, gulabjamun and khoya), paneer, cheese from different sweet vendors which were also considered environmental sample category. Raw milk was obtained from points of sale and collected in sterile recipients. The samples were transported in an isothermal box to the SEML laboratory of department of Microbiology, Assam University Silchar within 3 hours and processed within 24 hours of collection.

3.2.2. Selective Isolation of *S. aureus*:

All the swabs and food samples were placed in 10ml alkaline peptone water (annexure-II), vortexed for 10 seconds and incubated for 24 hours at 37 °C for enrichment. Following incubation, a loopful of inoculum was plated onto Mannitol Salt Agar (MSA, Hi-Media) (annexure-II) and incubated in ambient condition at 37 °C for 24-48 hours. Milk samples were inoculated onto the MSA plates and incubated at 37 °C in an incubator. The plates were examined after 24-48 h of incubation for bacterial growth and colony characteristics. Growth characteristics on MSA was recorded and interpreted as positive or negative based on standard procedure. Isolates that grew and fermented mannitol thereby producing yellow zones on MSA after 24-48 h incubation at 37 °C were tentatively considered *S. aureus*. MSA plates without typical *Staphylococcal* colonies after 24-48 hours of incubation were discarded.

The pre-dominent colony type per sample were further presumptively identified in accordance with established microbiological guideline (Bergey's manual). The taxonomic identification of the selected isolates were confirmed by amplification *16S rRNA* gene.

3.2.3. Mantainance of Stock Cultures:

For the mantainance of stock cultures, bacterial cells were grown on nutrient broth (NB) and stored at -50 °C in 40% glycerol. Cultures for experimentation were revived on nutrient agar (NA) and stored at 4 °C. Cells were transferred to fresh NA plates on weekly basis and fresh cultures were made from the stock cultures and kept at -50 °C for retrival. The stocks were maintained in SEML laboratory of department of Microbiology, Assam University, Silchar.

3. 3. Identification of *S. aureus*:

3. 3.1. Morphological Characterization:

The isolates were identified for their colony characteristics and cell morphology. Typical colonies of *S. aureus* with similar morphologies were isolated and cultured separately.

3.3.2. Biochemical Characterization:

3.3.2.1. Gram staining:

A drop of normal saline was placed on a well labelled clean grease-free glass slide using a sterile inoculating loop a colony of an overnight culture of the bacterial isolate was emulsified with the normal saline to make a thin smear. The smear was air dried and then heat fixed. The slide was flooded with crystal violet (primary stain) for 1min after which the stain was rinsed from the slide with water. The smear was flooded with Lugol's iodine to fix the primary stain. The iodine was rinsed with water after 1min**.** The slide was then flooded with a decolourizer (acetone) and rinsed off almost immediately**.** The counter stain; safranin

was added and left for 1min before being rinsed off. The stained smear was air dried, and then observed under the microscope at 1000X magnification. A cluster of purple colonies was indicative of staphylococci.

3.3.2.2. Catalase test:

A drop of 3% hydrogen peroxide was placed on a clean grease-free glass slide. About 2 colonies of the bacteria were picked from a culture plate using a sterile wire loop and placed on the hydrogen peroxide presence of bubbles indicated positive catalase test.

3.3.2.3. Coagulase test:

About one or two drops of blood plasma were placed on a clean grease-free glass slide and 2 colonies of the organism were picked using a sterile wire loop from a 24h old nutrient agar plate. The colonies were emulsified in the blood plasma and kept for incubation from 4-6 h. Formation of plasma clot indicated a positive coagulase test.

3.3.2.4. Oxidase test:

A piece of filter paper was socked in freshly prepared oxidase reagent. A single *S. aureus* colony was smeared on the filter paper properly using a glass rod. Appearance of blue purple colour within 10 s indicates positive result.

3.3.2.5. Indole test:

About 2-3 colonies of *S. aureus* were inoculated in a bottle containing 3ml sterile tryptone water and incubated at 37 °C upto 48 h. 0.5ml reagent was added and shaked gently. Appearance of red colour indicates positive result.

3.3.2.6. Nitrate reduction test:

About 2-3 colonies of *S. aureus* were inoculated in 0.5ml sterile nitrate broth and incubated at 37 °C for 24 h. 1 drop of each sulphanilic acid and alpha-napthylamine reagent was added and shaked gently. Appearance of red colour within 1-5 min indicates positive result.

3.3.2.7. Citrate test:

Slope of Simmons citrate agar was prepared in test tube (stored at 2-8°C). the slope was then streaked with a saline suspension of *S. aureus* and stabbed the butt. The tubes were incubated at 37 °C for 48 h. visible blue colour of the medium indicates positive result.

3.3.2.8. Methyl red test:

A single *S. aureus* colony was inoculated in 0.5ml sterile glucose phosphate broth and incubated overnight at 37 °C. A drop of methyl red reagent was added. Appearance of bright red colour indicates positive result.

3.3.2.9. Voges-Proskauer test:

About 2-3 colonies of *S. aureus* inoculated in 2 ml of sterile glucose phosphate peptone water and incubated at 37 °C for 48 h in a screw cap tube. 0.6ml α-napthol and 0.2ml 40% potassium hydroxide reagent was added and shaked vigorously with aeration. The bottle cap was removed and left for 1 h at room temperature. Appearance of pink red colour indicates positive result.

3.3.2.10. Alkaline phosphatase test:

A single *S. aureus* colony was streaked in phenolphthalein agar and incubated at 37 °C for 18-20 h. 0.1-0.2 ml ammonia solution was placed on the lid of the petridish and the medium was inverted above it. Appearance of bright pink colonies indicates positive result.

3.3.2.11. Urase test:

About 2-3 colonies of *S. aureus* were inoculated in a bottle containing 3ml sterile urea broth and incubated at 37 °C for 3-12 h. Appearance of pink colour of the broth indicates positive result.

3.3.2.12. Acid/gas from sugar:

About 2-3 colonies of *S. aureus* were inoculated in broth based sugar medium of respective sugar in screw cap tubes and incubated at 37 °C upto 7days and monitored for acid/gas production daily.

3.4. Antimicrobial susceptibility testing:

Saline suspension of isolated colonies was prepared from an 18-24 h old culture of isolates on MSA and used as inoculum. The suspension was adjusted to match the 0.5 McFarland turbidity standard, by suitably diluting in 0.9% NaCl solution. A sterile cotton swab was dipped into the adjusted suspension and excess inoculum was removed by pressing the swab firmly on the inside wall of the tube. The dried surface of a Müeller-Hinton agar plate was inoculated by streaking the swab over the entire surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The antimicrobial discs were placed firmly on the surface of the inoculated agar plate using sterile forceps. The plates were incubated at 35 °C overnight within 15 min after the discs were applied. After 16 to 18 h of incubation, the plates were examined and the diameters of the zones of inhibition were measured. Results were classified as susceptible, intermediate, or resistant, according to the approved guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2011).

3.5. Molecular Characterization of *S. aureus*:

3.5.1. DNA extraction for amplification:

The strains were further confirmed by amplification of *16S rRNA* gene using a set of primer pairs F-AGAGTTTGATCCTGGCTCAG and R-AAGGAGGTGATCCAGCCGCA**.** Genomic DNA was extracted from overnight cultures of *S. aureus* using the HiPurA Bacterial Genomic DNA Purification kit (Hi-Media) following manufacturer's instruction for Gram positive bacteria. Briefly, after cultivation of the isolates for 24 h at 37 °C on blood agar plates, 5-10 colonies of the bacteria were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8) containing 5 µl lysostaphin (1.8 U/µl). After 1 h incubation at 37 °C, 25 µl of proteinase K (8 mg/ml) and 200 µl of buffer AL (containing reagents AL1 and AL2) were added. The suspension was incubated for 30 min at 56 °C and for 10 min at 95 °C, and after a

spin for a few seconds an amount of 420 µl ethanol was added to each sample and placed in a spin column. After centrifugation for 1 min, the HiPurA spin columns were placed in a clean collection tube and the samples were washed twice with 500 µl of buffer AW. After a second wash and a centrifugation for 3 min, the HiPurA spin columns were placed in a clean 2 ml microfuge tube and the DNA was twice eluted with 200 µl and 100 µl of buffer AE, respectively. The purified DNA were stored at 4 °C for short term storage (24-48 h) and -20 °C for long term use.

3.5.2. Polymerase chain reaction (PCR):

PCR amplifications were performed in a total volume of 20µl made up of the genomic DNA,PCR water, primers and Red Taq Mastermix (Sigma-Aldrich, India). The Red Taq mastermix contained 0.2 units/ml Taq DNA polymerase, 32mM $(NH4)_2SO_4$, 130mM Tris HCl, 0.02% Tween 20, 3mM $MgCl_2$ and 0.4 mM dNTPs (dATP, dCTP, dGTP, dTTP) and inert red dye. The protocol for amplification was set in the PCR thermocycler (Bio-Rad, Germany) depending on the primers used. After the amplification, the PCR products were separated by agarose gel electrophoresis.

3.5.3. Agarose Gel Electrophoresis:

Agarose powder (Hi-Media) was used in preparing the agarose gels used in this study at 2% concentrations. The agarose was dissolved in 0.5 x TBE: Tris-Borate-EDTA and microwaved to dissolve the agarose and casted on gel plate putting the comb properly. The electrophoresis chamber was filled with the running buffer (0.5 x TBE) and casting tray was placed in the chamber removing the comb. The gel was totally submerged in the buffer. A tracking dye was added to the PCR products that were colourless to make them visible. The amplicon (10µL) was loaded into each well. A 100bp molecular weight marker was loaded into the first and the last well as a standard for estimating the size of the resulting DNA fragment. The electrophoresis chamber was connected to the power source and the DNA was

run at 80V for 30-45 min. The separated DNA fragments were visualized by staining the gel with ethidium bromide for 15 min and then de-stained in water for 15 min. The DNA bands were viewed by illumination with UV light and images recorded.

Two amplified products each from a clinical and a CA/environmental isolate was sequenced commercially (Xcelris, India). The sequences were compared with those available in the GenBank using Blast search. The sequences were subjected to nucleotide blast and >99% match score was accepted for the species identity. The sequences were submitted to the GenBank.

3.6. *In-vitro* biofilm formation:

3.6.1. Congo-Red agar method:

A qualitative screening of biofilm formation was determined by Congo red agar (CRA) method (Freeman *et al*., 1989). Preparation of Congo red (annexure-II) was done separately as concentrated aqueous solution and autoclaved at 121 °C for 15 minutes, and was then added to the sterilized agar at optimum temperature of about 55 °C. Plates were inoculated aseptically and incubated aerobically for 24-48 h at 37 °C in static condition. Positive result was identified by visible black colonies with a dry crystalline consistency whereas biofilm negative colonies usually remained pink. Darkening of the colonies with the absence of a dry crystalline colonial morphology specified an indeterminate result. The experiment was performed in triplicates and repeated three times.

3.6.2. Tube method:

Biofilm production was estimated quantitatively for all *S. aureus* isolates by tube method as described previously by Christensen *et al*. (1985). Test tubes were sterilized by autoclaving at 121 °C for 15 minutes prior to the experiment. An overnight culture of *S.*

aureus grown in peptone water (5ml) supplemented with 1% glucose and 0.75% NaCl incubated at 37 °C for 48 h. After incubation, the content of each tube was gently decanted and washed with sterile distilled water followed by PBS (pH=7.2) to remove loosely adherent free floating planktonic cells. The tubes were dried and stained with 0.1% (v/v) crystal violet for 10 min and then washed with distilled water followed by PBS, thrice to process biofilm for quantification. A positive result was specified by the presence of an adherent film of stained material on the inner wall of the substrate. Crystal violet stained biofilm was solubilized in 5ml of 70% ethanol for biofilm quantification. The OD of stained film were recorded at 620nm with 70% ethanol as blank in a spectrophotometer (Systronics visible spectro, 105N). Tube containing un-inoculated peptone water only was used as negative control. Experiments were performed in triplicate and repeated three times for all the isolates.

3.6.3. Determination of adherence value*:*

The adherence value was determined by using the following formula (Christensen at al., 1985) with a slight modification in the culture broth (peptone water instead of TSB broth).

$$\text{Adherence value} = \sqrt{A^2 + B^2}$$

Where, A= OD_{620} in peptone water with glucose

B =OD_{620} in peptone water without glucose

This formula calculated the distance between the origin and the adherence coordinates of a strain of coagulase-positive staphylococci, when the absorbance of the strains in peptone water and peptone water without glucose were plotted as the ordinate and abscissa, respectively.

3.7. Effect of different growth parameters in Biofilm Development:

3.7.1. Surface induced biofilm formation assay:

This experiment was conducted to figure out the role of different surface on induction of biofilm. The different surfaces considered were glass, polystyrene and PVC materials for

in-vitro biofilm growth. Standard test tube (borosil, 15X125mm) was used for glass material and polystyrene tubes (Falcon polystyrene, 14mL) were used as polystyrene experimental surfaces. Poly-vinyl chloride (PVC) material was tested in a specially designed experiment with modification of tube method for this particular trial. Experimental PVC materials were collected from a PVC pipe of 10mm diameter and fragmented to get several PVC chips (each 10X10mm). The PVC chips were soaked into acetone overnight in a glass beaker and finally exposed to UV-ray before experiment for sterilization. The sterilized PVC chips were fastened properly with a piece of sterile thread and inserted vertically or horizontally into the glass tube containing *S. aureus* cultures in peptone water broth (5mL) supplemented with 1% glucose and 0.75% NaCl. The stopper of the tubes were blocked up and settled for incubation at 37 °C and for 48 h to allow them to grow biofilm *in-vitro* (figure-1). After incubation, the content of each tube was gently decanted through the side of the tubes and washed with PBS thrice (pH=7.2). The tubes were air dried and stained with 0.1% (v/v) crystal violet for 10 min and washed again with distilled water followed by PBS to process biofilm growth. Positive result was detected by the adherence of a thin film of stained material on the inner wall of the surface. The stained film was solubilized in 5ml of 70% ethanol and optical densities (OD) were measured at 620nm with 70% ethanol as blank. Tubes containing peptone water only was used as negative control. The experiment was repeated in triplicates.

3.7.2. Physiological glucose concentration:

Varied glucose concentrations were also tested for its effect on *in-vitro* biofilm formation. *S. aureus* culture (~10^3 cfu) in peptone water broth supplemented with glucose [0%, 0.25%, 0.50%, 1% and 2% (w/v)] in glass tubes were assessed for its ability to form biofilm using the method described above (3.6.2. section). After culturing at 37 °C for 48h, biofilm formation was determined. Cultures without additional glucose were considered as controls.

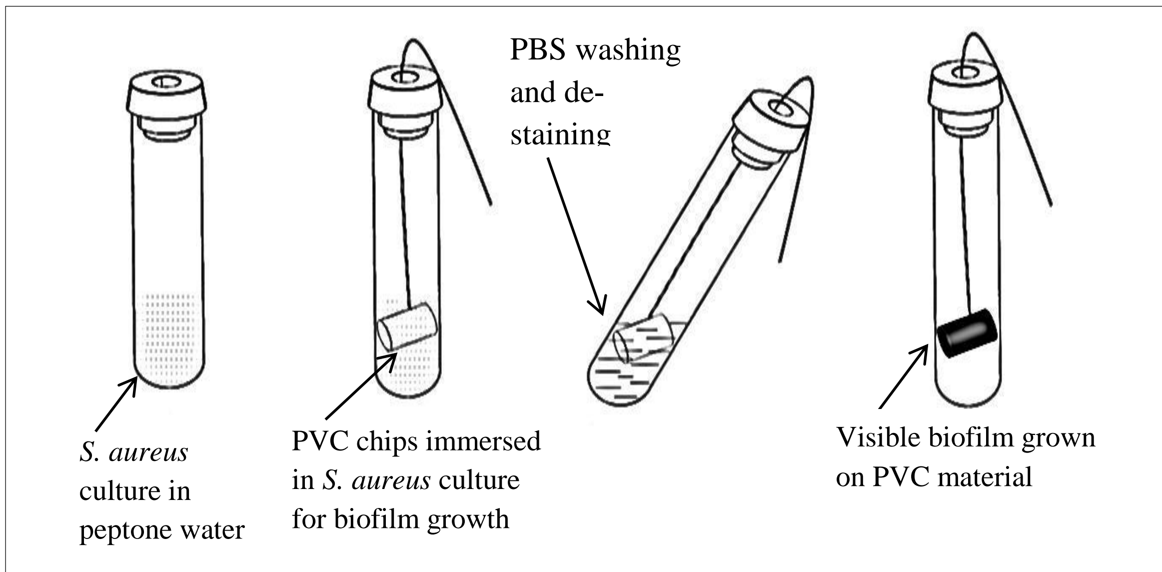

Figure 3.3. Diagrammatic representation of biofilm development in PVC material. (A) Test tube containing *S. aureus* culture in peptone water with 1% glucose. (B) PVC section fastened with sterile thread and immersed in the broth for 48 hrs at 37 ºC. (C) PBS washing thrice and stained with CV. (D) Visible biofilm growth over the PVC chip subjected to quantification in 70% ethanol.

3.7.3. Osmolarity:

NaCl was tested for its effects on biofilm formation. *S. aureus* (~10^3 cfu) cultured in peptone water supplemented with NaCl [0%, 0.25%, 0.50%, 0.75%, 1%, 1.5% and 2% (w/v)] after autoclaving was added to the biofilm broth. After culturing at 37 °C for 48h, biofilm formation were determined using the methods described above (3.5.2. section). Cultures without supplementary salts were considered as control.

3.7.4. Incubation Period:

Effect of incubation period was observed on formation of biofilm. *S. aureus* (~10^3 cfu) culture was grown in sterile peptone water and incubated at 37 C for variable durations (24h,

48h, 72h, and 96 h) to establish biofilm as described above (3.5.2. section) keeping all other parameters.

3.7.5. pH:

The pH of the peptone water broth was adjusted in the range 5, 6, 7 and 8, with 1M HCl or 1M NaOH. 0.1ml inoculum of log phase cells of *S. aureus* (~10^3 cfu) was inoculated into sterile peptone water broth in tubes with different pH. After 48h at 37 °C, biofilm formation under different pH conditions were measured as described earlier (3.5.2. section).

3.7.6. Antibiotic treatment (Oxacillin):

Peptone water (1mL) containing oxacillin (8mg/mL) were added to 24 h pre-established biofilms (as discussed earlier, 3.5.2) to determine the effect of oxacillin on biofilm. Control trial was processed in parallel where oxacillin free-peptone water was added to established biofilms. Biofilms were quantified at 620nm as described. Different OD values between antibiotic treated and antibiotic-free biofilms were plotted in a graph to trace the effect.

3.8. Microscopic analysis of biofilms:

The biofilm architecture and the mode of biofilm formation of *S. aureus* isolates were observed by Scanning Electron Microscpe (SEM, JSM-6390/LV) and Confocal Laser Scanning Microscope (CLSM, Olympus).

3.8.1. Scanning Electron Microscopy (SEM) :

Scanning Electron Microscopy was performed to assess the biofilm structure in presence and absence of oxacillin. Primarily, an oxacillin concentration of 8mg/mL was added to a 24 h pre-established biofilm in peptone water whereas biofilm without oxacillin was considered as control. Biofilms of *S. aureus* isolate C4075 was allowed to develop on 22×22 mm^2 glass coverslips (SAIL BRAND, China) in duplicates with late log phase bacterial culture (~10^3 cfu). Set- I was maintained with oxacillin treatment and set number-II was used as a control without oxacillin treatment. Primary fixation was done using 2.5% gluteraldehyde for 4 hours

followed by washing with 0.1M sodium cacodylate buffer for 1 h. A series of dehydration process was maintained from 30% to 100% each for 15 minutes for two changes and then immersed in tetra-methyl silane for 10 min at 4 °C and finally settled at 25-26 °C. Biofilms were mounted on aluminium stubs and sputter coating was continued with gold of about 35mm thickness. Images were viewed using a JSM-6390/LV scanning electron microscope for both the trial sets.

3.8.2. Confocal Laser Scanning Microscopy (CLSM):

Confocal Laser Scanning Microscopy was performed to study the biofilm behaviour of *S. aureus* isolates in presence and absence of oxacillin same as described above (3.7.6). A set of duplicate culture of *S. aureus* C4075 was designed for the experimentation. Set-I was added with 8mg/mL oxacillin antibiotic while set-II was used as control without oxacillin. A McFarland 0.5 standard suspension of *S. aureus* was prepared for each set in peptone water broth. Two test-tube containing a small glass disc in each was inoculated with 1 ml of each suspension and incubated for 24 h at 37 °C. At this stage 10µl oxacillin at 8mg/mL concentration was added to set-I and a re-incubation for 24 h at 37^0C was given. After incubation, the medium was removed and the tube containing disc was washed 3 times with sterile distilled water. The disc was then removed and stained with Live/Dead BacLight stain using the instructions provided by the manufacturer. Stained disc from both set was examined with a confocal laser scanning microscope (Olympus; 400X magnification) at 488nm excitation wavelength. The experiment was performed in triplicate and repeated 2–3 times for each set. The percentage of covered surface was calculated using ImageJ software.

3.9. Molecular detection of biofilm gene:

Determination of the *icaA, icaB, icaC* and *icaD* gene which codes for cellular adhesion resulting biofilm production was carried out as described (Booth *et al.* 2001). The sequences

were taken from the GenBank sequence database of the National Centre for Biotechnology Information. The reaction volume was 20 µL containing 1µL of each the forward and reverse primers together with 2µL of the extracted DNA, 10 µL of PCR Master Mix and 6 µL of distilled water. A PCR cycle for all the *ica* gene was programmed, considering initial denaturation at 95 °C for 5 min, followed by cyclic denaturation, annealing and extension of 50 cycles at 94 °C for 30 seconds, 55.5 °C for 30 s, and 72 °C for 30 s respectively followed by a final extension at 72°C for 1 min were performed in thermocycler (Eppendorf, USA). The amplicons were analysed on 2% agarose gel against 100 bp DNA marker (Hi-media).

For detection of *bap* gene the PCR conditions were the following: an initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, specific temperature of annealing for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR products were confirmed by agarose gel (2%) electrophoresis against 100bp DNA ladder.

3.10. Haemolysin Production:

3.10.1. Phenotypic detection:

Each *S. aureus* isolate were grown on the 5% sheep blood agar (annexure-II) plate as a spot inoculation and incubated for 24 h at 37 °C. Haemolysin production was confirmed by a clear zone around the colonies and the types of haemolysins were characterized based on the zone of lysis produced by the isolates. The diameter of haemolytic zone for each isolate was measured and recorded. Haemolysin activity was further continued by amplifying the genes encoding for haemolysins, *hla* genes for alpha haemolysin and *hlb* genes for beta haemolysins using PCR.

3.10.2. Detection of haemolysin gene:

Amplification reactions with PCR were perfomed according to the references for *hla* and *hlb* genes (Booth *et. al.,* 2001). The reaction volume was 20 µL containing 1µL of each the forward and reverse primers together with 2µL of the extracted DNA, 10 µL of PCR Master Mix and 6 µL of distilled water. The PCR cycle involved: initial denaturation at 95 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. PCR products were then separated by gel electrophoresis in a 2% w/v agarose (Hi-media) gel run in 0.5 × TBE buffer. A 100 bp DNA ladder (Hi-Media) was used as a size marker.

3.11. Molecular detection of enterotoxin gene:

Sequences specific for enterotoxins; *sea* and *sed* detected by PCR as described (Lovseth *et. al.,* 2004). The priimers were listed in table. The reaction volume was 20 µL containing 1µL of each the forward and reverse primers together with 2µL of the extracted DNA, 10 µL of PCR Master Mix and 6 µL of distilled water. The optimized thermal cycling conditions for the multiplex PCR were denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and a 5 min extension at 72 °C after the last cycle. Amplified products were resolved by gel electrophoresis on 2% agarose and the bands were visualized under UV illumination.

Table. 3.1. Lists of primers used.

Primers used	Sequence	Product size (bp)	Reference
icaA	F- TCTCTTGCAGGAGCAATCAA R- TCAGGCACTAACATCCAGCA	188	Booth *et al.,* (2001)
icaB	F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTTCATGGAATCCGT	900	Atshan *et al.,* (2012)
icaC	F- ATGGGACGGATTCCATGAAAAAGA R- TAATAAGCATTAATGTTCAATT	1100	Atshan *et al.,* (2012)
icaD	F- ATGGTCAAGCCCAGACAGAG R- CGTGTTTTCAACATTTAATGCAA	198	Booth *et al.,* (2001)
bap	F-CCCTATATCGAAGGTGTAGAATTGCAC R- GCTGTTGAAGTTAATACTGTACCTGC	971	Cucarella *et al.,* 2001
hla	F- GGTTTAGCCTGGCCTTC R- CATCACGAACTCGTTCG	550	Booth *et al.,* (2001)
hlb	F- GCCAAAGCCGAATCTAAG R- GCGATATACATCCCATGGC	840	Booth *et al.,* (2001)
sea	F- GCAGGGAACAGCTTTAGGC R- GTTCTGTAGAAGTATGAAACACG	521	Lovseth *et al.,* (2004)
seb	F-GTATGGTGGTGTAACTGAGC R-CCAAATAGTGACGAGTTAGG	164	Mehrotra, *et al.,* (2000)
sec	F-AGATGAAGTAGTTGATGTGTATGG R- CACACTTTTAGAATCAACCG	451	Mehrotra, *et al.,* (2000)
sed	F- GTGGTGAAATAGATAGGACTGC R- ATATGAAGGTGCTCTGTGG	385	Lovseth *et al.,* (2004)

3.12. Detection of extracellular virulence factor:

3.12.1. Protease Production:

Casein agar prepared by supplementing nutrient agar with 2% casein (Sigma) (annexure-II) was used to screen for protease activity. The isolates were sub-cultured on BHI agar and incubated at 37 °C for 72 h. After incubation, the isolates were inoculated on casein agar plates and again incubated at 37 °C for 72 h. The isolates producing opalescent zones around the colony were confirmed as protease positive (Saising *et al.,* 2012).

3.12.2. Production of thermostable nuclease (TNase):

Several colonies were inoculated into 1mL of the BHI broth (annexure-II) and incubated at 37 °C for 2h followed by heat suspension at 100 °C for 15min and then allowed to cool to room temperature. In a plate of the toluidine blue DNA agar, 6mm diameter wells cut (using blunt end of a sterile cutter, maximum 12 wells per plate) and each well was filled with the cooled broth suspension. The plates were then incubated at 35-37 °C and examined hourly for up to 4hr. Pink zone of clearing at the edge of the well with a darker blue ring at the outer periphery of the zone indicate positive result.

3.12.3. Lipase Production:

Tributyrin (Fluka) agar plates (annexure-II) were used to study lipolytic activity. The isolates were sub-cultured on BHI agar and incubated at 37 °C for 24 h. After incubation a single colony was inoculated on tributyrin agar plates and incubated at 37 °C for 72 h. The presence of clear zones was taken as an indication of positive lipase activity (Saising *et al.,* 2012).

3.12.4. Lecithinase Production:

Egg yolk agar plates (annexure-II) were prepared to screen lecithinase producing *S. aureus* isolates by adding egg yolk emulsion to nutrient agar. *S. aureus* isolates were inoculated as 2-3 diameter spot inoculation onto egg yolk agar plate and incubated for 72 h at

35 °C. Isolates demonstrating opaque growth were considered lecithinase positive (Baron *et al.,* 1994).

3.13. Statistical analysis:

Prism 6 and SPSS 18 were used for the purpose of statistical analysis. All the clinical and environmental *S. aureus* isolates were compared using the analysis of variance (ANOVA), F-test, Pearson chi-square test. *P* values of <0.05 were considered statistically significant.

____