CHAPTER-4

RESULTS

4. 1. Preliminary Screening:

4.1.1. Identification of Isolates

The samples were collected for the study during March 2012 to February 2013 from different environmental and clinical samples from the mentioned study area revealed prevalence of *S. aureus* in those samples. In the present study a total of 872 clinical and environmental samples were collected from three districts covering South Assam region of India. Out of 872 samples, 527 were from clinical source and 345 were from environmental origin. The clinical isolation of *S. aureus* considering different parameters are given in table 4.1. In short, the frequency of *S. aureus* isolation is most prevalent from patient of 80 years and above (88%), females (54%), urban locality (75%) and nasal swab (78%) when considered demographic factors like age, sex, locality and sample source. Among the total 527 clinical samples, 264 isolates (50%) were found to be positive. On the otherhand, 180 isolates (52.17%) out of 345 community associated environmental samples were found to be positive. The sample source and frequency of positive isolates in environmental samples are given in table 4.2, which depicts the high prevalence of *S. aureus* in normal skin micro-flora (80%), raw milk (65%), and paneer (64.5%). Out of 872 samples, on the basis of preliminary screening a total of 444 (50.91%) consisting 264 clinical and 180 environmental samples were found to be positive for *S. aureus*. The results of preliminary screening are given in table 4.3.

4.1.2. Morphological and cultural characteristics

Under microscope, almost all *S. aureus* colonies appear Gram positive, spherical cocci in cluster, small to medium sized, non-motile, non-spore forming.

Cultural characteristics showed that all the colonies were circular in shape and shiny to opaque in appearance. The colonies of the suspected Staphylococcal isolates formed golden yellow halo on mannitol salt agar medium due to fermentation of mannitol and appeared

golden yellow after incubation of 24-48h at 37 °C. When grown on blood agar media containing 5% sheep blood, it appeared as white to golden colony. The cell elevation was mostly raised and convex in shape and rough in nature. The cell diameter was recorded as 1-6mm. Phenotypical analysis showed two varieties of *S. aureus*, the wild type (WT) and small colony variant (SCV). The frequency of WT and SCV in clinical isolates were 72% and 28% respectively whereas, environmental isolates revealed a prevalence of 67.2% WT and 32.8% SCV phenotypes. Therefore among total 444 isolates, 311 (70%) isolates were WT and 133 (30%) isolates were SCV phenotype (table 4.4). The majority of the time the colony produces a zone of haemolysis (beta-haemolysis) surrounding the colony. The colony formation of an WT isolate SAC11 and SCV isolate SAE61 in MSA medium is shown in figure 4.1 and figure 4.2 respectively.

4.1.3. Biochemical Characteristics

On the basis of Gram staining a total of 488 (55.96%) isolates were suspected to be positive (Figure 4.3). Further presumptive biochemical identification tests showed a positive result for catalase, coagulase, and mannitol fermentation.

Catalase positive isolates breaks down H_2O_2 to O_2 and H_2O producing bubbles of O_2. Immediate bubbling interpreted the positive result. Out of 872 samples, 457 (52.40%) samples were found catalase positive (Figure 4.4).

Coagulase positive isolates causes fibrin of blood plasma to clot. Clot formation within 4h-24h interpreted positive result. A total of 444(50.91%) among 872 samples interpreted positive coagulase test (Figure 4.5). Negative test isolates were discarded.

Positive isolates breaks mannitol sugar of MSA medium to produce acid which lowers down the pH of the medium and changes colour from red to yellow. Formation of yellow halo around colonies inferred positive mannitol fermentation. It was observed that 444 isolates (50.91%) out of 872 samples produces acid by fermenting mannitol. Therefore a total

of 444 isolates out of 872 samples showed similar result for coagulase positive and mannitol fermentation and thus confirmed as positive *S. aureus* (Table. 4.3). The other biochemical confirmatory tests are given in figure 4.6 and table 4.4.

4.1.4. Molecular identification and sequencing

Two *S. aureus* isolates, one from clinical (SaC51) and one from environmental isolate (SaE34) were sequenced on the basis of their *16SrRNA* gene followed by nucleotide homology and phylogenetic analysis. The sequences were submitted to NCBI Gen-bank and accession number obtained for the two isolates. The sequences were first analysed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to study the closest homologous sequences. The relevant sequences were saved in FASTA format (*.txt) and processed in MEGA-5 software for phylogenetic tree. The distance matrix was prepared by using Kimura-2 parameter and finally phylogenetic tree was constructed by using Neighbour joining method (Table 4.5, Figure 4.7).

The nucleotide sequence of *16SrRNA* of clinical isolate (Sac51) is as shown:

TTTTATGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGA
CGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGAT
AACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTG
TCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCG
ACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTAT
TAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTT
TTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGG
AAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGG
TCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAA
AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCGAAACC
GCGAGGTCAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAA
TCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTATTGTACACACCGCCCGTCACACCACG
AGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCTTTTAGGAGCTAGCCGTCGAAGGTGGGACAAATGATTGGGG
TGAAGTCGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCT

The nucleotide sequence of *16SrRNA* of environmental isolate (SaE34) is as shown:

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCG
GCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGG
ATAATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATT
AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTG
TGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTC
AACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAA
ATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGC
GTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGA
CATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGG
GCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATT
TGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTG
TTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTA
CGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGA
GTAACCTTTTAGGAGGGTGGGACAAATGATTGGGGTGAAG

The sequence analysis of *16S rRNA* of clinical isolate Sac51 showed a 1548 base pair encoding 516 amino acids. The result of the nucleotide Basic Local Alignment *Sea*rch Tool (BLASTn) showed that the clinical strain Sac51 had highest homology (100%) with *S. aureus* strain ATCC 12600 (Genbank Accession Number NR_118997.1). However, the sequence analysis of *16S rRNA* of environmental isolate SaE34 showed a 1465 base pair encoding 488 amino acids. BLASTn showed that the strain SaE34 had highest homology (100%) with *S. aureus* strain FCFHV36 (Genbank Accession Number CP011147.1).

This therefore confirms that the isolates that showed amplification with *16SrRNA* genes are actually *S. aureus.* The relationships among species from both the origin of the genus *Staphylococcus* were confirmed by phylogenetic analysis based on the *16SrRNA* gene sequencing, and the topology of the tree was evaluated by bootstrap values.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length is calculated as 3.01514102. The evolutionary distances

were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Figure 4.7 represented the phylogenetic tree showing the lineage of the clinical strain (Sac51) and environmental strain (SaE34) with the other *S. aureus* strains, interspecific *S. epidermidis* strain and an outgroup strain *P. aeruginosa*.

4.2. Antimicrobial Susceptibility Profile of *S. aureus* isolates

The antimicrobial susceptibility pattern of 444 *S. aureus* strains isolated from various clinical and environmental origin are given in table 4.6 and 4.7 respectively. Among the clinical isolates, both vancomycin and linzoid exhibited highest sensitivity (100%) and this was followed by oxacillin (63%), ciprofloxacin (54%), amikacin (45%), ceftriaxone (43%), cefuroxime (43%), erythromycin (38%), tetracycline (35%) and gentamycin (10%). Clinical isolates showed zero sensitivity and highest resistance (100%) against Penicillin and ampicillin antibiotic followed by gentamycin (69%), cefrtriaxone (46%), tetracycline (40%), cefuroxime (33%), erythromycin (27%), amikacin (25%), oxacillin (23%) and ciprofloxacin (20%).

On the otherhand, the resistance rate of environmental isolates were maximum against ampicillin (100%) and penicillin (96%). This was followed by gentamycin (56%), tetracycline (41%), ceftriaxone (29%), oxacillin (25%), cefuroxime (23%) and erythromycin (20%). Both amikacin and ciprofloxacin showed similar resistance pattern (15%). Vancomycin and linzoid also exhibited similar sensitivity pattern with clinical isolates and stand for maximum sensitivity (100%), followed by oxacillin (65%) and cefuroxime (54%). Ciprofloxacin and ceftriaxone showed similar sensitivity pattern (51%). Amikacin showed 45% sensitivity followed by tetracycline (28%) and gentamycin (20%). The last sensitivity

was observed in case of penicillin 4% and finally ampicillin showed no sensitivity at all. Of all the *S. aureus* isolates, 23% in clinical and 25% in environmental isolates exhibited methicillin resistance. However there was 100% susceptibility to glycopeptides (vancomycin) and oxazolidinones (linzoid) antibiotics in both the isolates. Thirty six (36) clinical isolates were susceptible to all antibiotics tested except penicillin. In contrast, thirty one (31) environmental isolates were found to be susceptible to all antibiotics. The prevalence of MRSA and MSSA in WT and SCV phenotypes are shown in table 4.8. Briefly, clinical SCV MRSA (4.2%) were more prevalent than environmental SCV MRSA (3.3%), whereas in WT isolates, environmental MRSA (21.6%) was more prevalent than clinical MRSA (18.9%). Figure 4.8 displayed the photoplate of a clinical isolate C1647 presenting antibiotic susceptibility test by Kirby-Bauer's disc diffusion method showing sensitivity against vancomycin (10µg) oxacillin (5µg) cefuroxime (30µg) and amikacin (30µg) and resistance against penicillin (10IU). The region-wise prevalence of MRSA, MSSA, WT, SCV phenotype of both clinical and environmental isolates were presented in figure 4.41.

The multiple antibiotic resistance (MAR) indexes of both clinical and environmental *S. aureus* isolates are shown in figure 4.8. Only 11% of the environmental isolates had 0.0 resistance index while 21% of the clinical isolates had 0% resistance. The remaining isolates had MAR index ranging from 0.1 to 1.0 indicating multiple resistance. However, most species (23.8%) had MAR index of 0.6 followed by 19.0% with 0.5. The antibiotic resistant of clinical *S. aureus* isolates have MAR index of 0.1 to 0.7, while the antibiotic resistant of environmental isolates have MAR index of 0.1 to 0.8. Calculated MAR index (Figure 4.9) suggests that almost all the test organisms exhibited multiple antibiotic resistance in the following order: *S. aureus* (environmental) > *S. aureus* (clinical), though there is no significance difference between the MAR indexes of environmental and clinical *S. aureus* samples ($p>0.05$).

4.3. Biofilm assay:

Among clinical isolates, 32.2% were high biofilm producer when assessed by TM while 20.8% were found high producer of biofilm in CRA method. Non-biofilm producers were 13.6% and 37.9% by TM and CRA method respectively. Moderate biofilm producer were found to be 25% in case of TM and 22.7% in case of CRA. Weak or low biofilms were observed as 29.2% in TM and conversely 18.6% in CRA method. Negligible, biofilm varied from 13.6% - 37.9% whilst 57.2% - 43.5% of isolates produced fully established biofilms as assessed by TM and CRA methods respectively (Table. 4.9). *In-vitro* biofilm production in CRA plate and TM are shown in figure 4.10 and figure 4.11 respectively.

Among clinical isolates, 151 (57%) isolates showed a good biofilm production (SA and MA biofilm) by TM but this amount is relatively low when assessed by CRA method where only 115 (44%) isolates showed good biofilm formation. In contrast, 100 (56%) and 83 (46%) environmental isolates showed good biofilm production (SA and MA biofilm) by TM and CRA method respectively (Table 4.9).

Environmental isolates also exhibited appreciable biofilm formation as determined by both methods. High biofilm production were observed as 24.4% and 16.7% whereas, 25.6% and 38.3% were non-biofilm producers determined by TM and CRA method respectively. On the other-hand, 36.7% and 29.4% isolates were moderate biofilm producer while 18.9% and 15.6% isolates were weak biofilm producers by TM and CRA methods respectively. In addition twelve (12) environmental MRSA isolates: EP44, ERM4, MC32, SK8, SK11, EC51, MHH45, EP8, EPM24, EC31, EC9, ECII were found to be high producer of biofilm (OD_{620nm} = 0.450- 0.770) by quantitative biofilm estimation by TM.

Phenotypically total biofilm formation by all the *S. aureus* isolates (n=444) were 82% whereas 18% isolates did not produce biofilm *in-vitro*. Among the biofilm producers, 29% were strongly adherent, 27.5% were moderate and 25% were least adherent (Table 4.10).

Quantitative tube method of biofilm screening showed the isolates were positive for biofilm production. Most of the isolates showed thick straw-coloured ring at the liquid-air interface. The optical density (OD) of non-adherent (NA), weak adherent (WA), moderate adherent (MA) and strong adherent (SA) obtained when the bacteria were grown in peptone water at 37 ^{0}C for 72hrs/3days ranged from 0.136–0.211, 0.310–0.408 and 0.481–0.879 respectively found to be maximum having mean OD±SD, 0.188± 0.15, 0.341± 0.07 and 0.678± 0.51 respectively whereas the OD values of NA category was found lower than control (LTC) and hence OD was not determined (ND). Data are shown in (Table 4.11).

Tube method was found to be most sensitive (96.1%) and specific (91.6%) with high accuracy in case of clinical isolates and 92.5% and 95.7% in case of environmental isolates respectively. CRA method showed very little correlation with corresponding tube method and the parameters of sensitivity and specificity were very low (78.6% and 88% respectively) in case of clinical isolate and environmental isolates (79.3% and 81.2% respectively). Results are given in table 4.12.

The difference in adherence coordinates for a total of 100 isolates comprising clinical (n=70) isolates and environmental (n=30) isolates were shown in Figure 4.12 and the correlation of adherence values between both clinical and environmental isolates, are shown in figure 4.13. The average adherence found to be superior in clinical isolates.

4.3.1. Surface adherence of biomaterials

Biofilm formation and adherence ability was demonstrated in isolates at different surface materials i.e., glass, polystyrene and PVC respectively. It was observed that biofilm formation was relatively higher on PVC material followed by polystyrene and glass surfaces.

PVC supported maximum biofilm production (41.44%), whereas glass and polystyrene supported for only 25.90% and 32.65% biofilm growth respectively (Table 4.13, Figure 4.14). The biofilm formation in PVC material was evident at 0.460±0.19 (mean OD±SD) in case of clinical isolates and 0.400±0.17 (mean OD±SD) in environmental isolates. Biofilm formation by SCV isolates also showed maximum growth on PVC surfaces (Figure 4.15). SEM analysis on different biomaterial surfaces also correlated maximum colonization of *S. aureus* biofilm in the case of PVC, followed by Polystyrene and glass. [Figure 4.16 (D), (C), (A,B)].

4.3.2. Effect of glucose concentration on biofilm production

Under 1% physiological glucose concentration 82% of the isolates showed maximum biofilm production *in-vitro* by TM. At 0% glucose concentration, least biofilm production was observed (18%). Further increase in glucose concentration to 1%, the amount of biofilm was found to be significantly more as compared to control ($p<0.001$). It was observed that total biofilm formation in glass surface was 22.08% and 3.82%; in polystyrene surface 27.70% and 4.95% and in PVC surface 31.53% and 9.91% in presence and absence of glucose respectively (Table. 4.13). Further, it was also found that 2% glucose concentration had an antagonistic effect on biofilm growth (Figure 4.17). Similar results were obtained with SCV isolates where maximum biofilm was formed at 1% glucose. 87% of SCV type isolates produced maximum biofilm at 1% glucose. (Figure 4.18). Figure 4.19 showed biofilm formation in WT isolates.

4.3.3. Effect of osmolarity (salt) on biofilm growth

Beside glucose, sucrose and galactose, the osmotic agent NaCl, was also chosen and evaluated for its effect on biofilm formation in *S. aureus*. The results illustrated that biofilm formation was generally inhibited at higher concentrations of NaCl (Figure 4.20, 4.22). Across a range of NaCl concentrations (0% - 2 %), biofilm growth was maximal at 0.75%.

The mean OD±SD found to be 0.420±0.09 and 0.380±0.11 in clinical and environmental isolates respectively. In contrast, biofilm formation was significantly repressed at higher concentrations of NaCl, especially at 2%. This observation suggests that NaCl positively affect biofilm formation through an osmotic ion effect. On the otherhand, biofilm formation in SCV isolates of both environmental and clinical origin also affected by NaCl concentration. An NaCl concentration of 0.75% showed maximum biofilm growth and also 1% NaCl found to be suitable for biofilm formation in SCV isolates (Figure 4.21).

4.3.4. Effect of incubation period on biofilm growth

S. aureus isolates displayed four different biofilm phenotypes (NA, WA, MA and SA) after incubation for 72 h in peptone water on different substrates (Table 4.10). It was observed that biofilm positive isolates adhered to the substrate wall when incubated for 72h (100%), irrespective of the substrate (glass, polystyrene and PVC). When the biofilm broth were incubated at 37 °C for 72h all biofilm producers (mean OD_{620nm}= 0.678 in SA type) were able to form biofilm as compared to 24 h, 48 h and at 96 h a remarkable declination of biofilm growth was observed (Figure 4.23). Initially biofilm formation was not significant at 24 h but an addition of another 24 h incubation was proved to be beneficial for biofilm development. In contrast, the maximal biofilm formation in peptone water biofilm medium, appeared on 72 h peak (mean OD±SD= 0.420±0.31 and 0.570±23 in clinical and environmental isolates respectively) and the biofilm growth was evident followed by a significant decline by the 96 h/4th day. However, some isolates (SAC31, SAC160, SAC109, Sap32, SaUII, SAN2, Sap81, Sas99, Sau70, SAO91) showed a good biofilm density at 96 h/4th incubation (Figure 4.24). SEM micrograph showing biofilm formation at different incubation period are given in figure 4.25.

4.3.5. Effect of pH on biofilm growth

Though *S. aureus* can grow in a broad pH range, however extreme acidic and alkaline pH inhibited biofilm growth. Interestingly, biofilm formation declined with increasing acidity of the medium at 37 °C in PW medium (Figure 4.26). An optimal pH for biofilm production in both clinical and environmental isolates was found to be pH=7.2. At this pH level the biofilm formation varied in clinical and environmental isolates were 0.400±0.13 and 0.390±0.15 (mean OD±SD). At pH=5, a decreased biofilm formation was marked among all the tested strains (mean OD±SD= 0.182±0.06). Further increase in pH= 6 showed a gradual steep in biofilm density and attains maximum at pH=7 and then gradually declined at pH=8 (mean OD±SD= 0.220±0.07). Among clinical isolates, biofilm formation in 2 isolates (Sa31, EC53) were found less than control at pH=6 but attained considerable growth at pH=7.2. Similar results also shown by WT and SCV isolates and maximum biofilm was formed at pH=7.2 though biofilm formation in SCV isolates was more significant than WT ($p<0.005$), (Figure 4.27) and the mean OD± SD was recorded as 0.460±0.17. Thus, a neutral to slight alkaline environment is superior for biofilm formation in *S. aureus*.

4.3.6. Effect of oxacillin on biofilm production:

Oxacillin was found to effect the production of biofilm in both clinical and environmental isolates (Figure 28a). Effect of oxacillin was studied on biofilm formation by clinical and environmental isolates using sub-lethal doses of oxacillin. In clinical isolates, it was noted that the oxacillin treated isolates showed 1.5 fold increase in biofilm production wrt control whereas 1.2 fold increase in biofilm production was evident in environmental isolates wrt control. Effect of oxacillin was also analysed in comparative MSSA and MRSA variants. In general MSSA variants were found to be higher producer of biofilm considering the average biofilm production by all the isolates. Relatively the amount of biofilm released was lesser in MRSA variants. Interestingly, the average value of biofilm production in

oxacillin stress condition was increased among MRSA and the increase was notably higher as compared to MSSA variants (Figure 28b).

Figure 4.29 (C), (E) shows a time-course study on the effect of oxacillin antibiotic (8mg/ml) after its exposure and (B),(D) before exposure. As expected, oxacillin was not very effective in killing biofilm bacteria. In fact, as compared with the untreated image, significant difference found at 24 h of exposure. Oxacillin stimulated biofilm growth as weak or loosely adherent biofilm producing isolates (OD_{620} <0.12) became moderately strong biofilm producers (0.12 ≤OD_{620} < 0.344) (Figure 4.23b).

There is a significant correlation between biofilm formation under control and oxacillin stress (p =0.0130), (r =0.318) in case of clinical isolates. Biofilm formation in environmental isolates under control and oxacillin stress also followed a significant correlation (p<0.0001), (r = 0.555).

4.4. Image Analysis:

Both SEM and CLSM imaging experiments were carried out to observe the effects of antibiotic (oxacillin) on *S. aureus* biofilm topography and architecture. Figure 4.29 shows a representative field of view obtained for isolate C4075. SEM and CLSM study, showed the comparative biofilm formation before and after exposure to oxacillin at 8 mg/mL. The addition of antibiotic (oxacillin) to 24h/1day old pre-established biofilm resulted in more abundant biofilm architecture [Figure 4.24 (C) and (E)] as compared to control [Figure 4.29 (B) and (D)]. It was observed that oxacillin stimulated production of biofilm matrices as evident by fluorescent green under CLSM compared with the sparse biofilm matrix of the control. The production of biofilm polysaccharide [Figure 4.29 (C) and (E)] and the multiplication of living cells attached to the surface were clearly visible. However, SEM study also elucidated the same results as CLSM study revealed and the thick or overgrowth of

polymeric matrix was witnessed in oxacillin treated biofilm as compared to control [Figure 4.29 (B) and (D)]. SEM micrograph of a clinical *S. aureus* isolate (C4075) and a single colony of SCV isolate (SaC64) are shown in Figure 4.30 and 4.31 respectively.

4.5. Molecular analysis of biofilm production:

4.5.1. Prevalence of biofilm genes (*icaABCD*) in clinical and environmental isolates

Among the total 444 *S. aureus* strains isolated from study area (Cachar, Karimganj, and Hailakandi) the prevalence of *icaA* and *icaD* gene were 59% and 47% respectively. However, 24.3% of total isolates showed presence of both *icaA* and *icaD* gene concomitantly [Figure 4.32) and (4.33), Table 4.14].

Among clinical isolates, *icaA* gene was most prevalent (61.4%) than *icaD* gene (39.4%) whereas, among environmental isolates, *icaD* (58.3%) gene was slightly more prevalent than *icaA* gene (55.6%). However *icaB* and *icaC* genes were absent in both clinical and environmental isolates. Although there is no significant correlation between clinical and environmental isolates ($p=0.0602$, $r=0.9398$) but the two genes *icaA* and *icaD* had a significant association between themselves ($p= 0.0053$).

Among 264 isolates from clinical origin of Cachar (n=90), Karimganj (n=76) and Hailakandi (n=98) district of south Assam, the region-wise prevalence rate of *icaA* genes were 15.56%, 31.84% and 13.18% and *icaD* genes were 11.78%, 14.52% and 13% respectively in clinical isolates. Whereas, prevalence of *icaA* genes were 17.04%, 25.4% and 12.5% and *icaD* genes were 25.6%, 13% and 18.6% in CA/environmental isolates of Cachar (n=71), Karimganj (n=67) and Hailakandi (n=42) respectively. A comparative data is shown in Figure 4.45. Frequency of *icaA* and *icaD* gene are shown in figure 4.38.

4.5.2. Prevalence of biofilm genes (*bap*) in clinical and environmental isolates

Among the total 444 *S. aureus* strains isolated from study area (Cachar, Karimganj, and Hailakandi) the prevalence of *bap* gene was 3.2%. However, *bap* gene was more prevalent in environmental isolates (5%) than clinical isolates 1.9% (Table. 4.13).

The region-wise prevalence rate of *bap* genes were 0%, 1.2%, 0.7% isolated from clinical samples and 02%, 03% and 0% isolated from environmental samples from the *S. aureus* isolates of Cachar, karimganj and Hailakandi districts respectively. Results are shown in Figure 4.45.

4.6. Haemolysin assay:

4.6.1. Phenotypic detection of haemolysis

Out of 264 clinical isolates, 50.76% isolates showed β-haemolysis, 28.3% MSSA and 22.7% MRSA isolates were found to be haemolysin producer. 28.78% isolates were α-haemolytic on 5% sheep blood agar plate among which 18.56% were MSSA and 10.22% were MRSA. Further 20.45% isolates showed no haemolysis at all. 79.53% clinical isolates were found to be haemolytic (Figure 4.34).

Among 180 environmental isolates, 56.11% showed β-haemolysis, out of which 41.11% MSSA and 15% MRSA isolates were found to be haemolysin producer. 11.11% isolates were α-haemolytic on 5% sheep blood agar plate among which 7.22% were MSSA and 3.89% were MRSA. In addition 32.8% isolates did not show any type of haemolysis and 67.22% isolates showed total haemolysis in environmental isolates (Figure 4.35).

4.6.2. Prevalence of haemolysin genes in clinical and environmental isolates

Among the total 444 *S. aureus* strains, the prevalence of *hla* and *hlb* gene were 45% and 50% respectively. However 16.7% of total isolates showed presence of both *hla* and *hlb* gene concomitantly [Figure 4.36, Figure 4.37, Table 4.15].

Among clinical isolates (n=264), *hlb* gene was most prevalent (31%) than *hla* gene (25.6%) whereas, among environmental isolates (n=180), *hla* gene (20%) gene was slightly more prevalent than *hlb* gene (19%). There is no significant correlation between clinical and environmental isolates ($p>0.005$) as well as between hla and hlb gene among all the isolates ($p>0.005$). Frequency of *hla* and *hlb* gene are shown in figure 4.39.

The region-wise prevalence rate of *hla* and *hlb* genes were 11.3%, 13.58% and 21% and 21.63%, 20% and 9.8% isolated from clinical samples and 7.89%, 16.4% and 20.3% and 16.2%, 21%, 10.76% isolated from environmental samples from the *S. aureus* isolates of Cachar, karimganj and Hailakandi districts respectively. Results are shown in Figure 4.45.

4.7. Prevalence of enterotoxin genes in clinical and environmental isolates

The enterotoxin producing ability of isolates was confirmed by amplification of enterotoxin gene (*sea*, *seb*, *sec* and *sed*) by standard procedure. Among a total of 444 *S. aureus* isolated from different clinical and environmental samples of south Assam, showed high prevalence of enterotoxin gene *sea* and *sed* whereas, *seb* and *sec* genes were absent in both clinical and environmental isolates. The prevalence of *sea* (Figure 4.40) and *sed* (Figure 4.41) gene were 65% and 45% respectively among all the isolates. However, 35% isolates showed presence of both genes concomitantly and 21% isolates did not carry either of the two genes.

Among clinical isolates (n=264), *sea* genes was most prevalent (35.01%) than *sed* gene (30.16%). Similarly in environmental isolates (n=180) too, the prevalence of *sea* gene

(29.65%) was more than *sed* gene (15.35%) (Table 4.16). Although there is no significant correlation between clinical and environmental isolates (p=0.055, r=0.9453) but an F test signified significant correlation between *sea* and *sed* genes among all the isolates (p= 0.006).

The area-wise prevalence rate of *sea* and *sed* genes were 16.40%, 27% and 14.7% and 19%, 16.4% and 14.7% from the *S. aureus* isolates of Cachar, Karimganj and Hailakandi regions respectively isolated from clinical sources. Prevalence of *sea* genes were 16.8%, 28.6% and 29% and *sed* genes were 7.23%, 12.2% and 18.10% from the *S. aureus* isolates of Cachar, Karimganj and Hailakandi regions respectively isolated from environmental samples. The results are given in figure 4.45.

4.8. Extracellular enzyme as virulence factor:

4.8.1. Prevalence of protease production

Seventy six (76%) isolates were found to produce protease as indicated by the zone of clearance around the colony on tributyrin agar plates. Among the total isolates 33% isolate showed the maximum zone of clearance (15-20mm) followed by 19% , 11%and 6% having (10-15)mm, (5-10)mm and (>20)mm zone of clearance. Only 7% isolates showed least zone of clearance (<5) mm as shown in figure 4.43. However, among the total isolates, clinical isolates (46.3%) were most prevalent in terms of protease production than the environmental isolates (30%) isolates (figure 4.42).

4.8.2. Prevalence of thermonuclease (TNase) production

Ninety four (94%) isolates were found positive for thermonuclease production while 6% isolates did not produce thermonucleases (SCV isolates of both clinical and environmental origin). Among the total isolates 32% isolate produced maximum clearance zone (10-15mm) followed by 26% and 18% isolates having (15-20) mm and (5-10) mm clearance zone

respectively. Only 9% isolates showed extreme clearance zone (<5mm and >20mm) as shown in figure 4.43.

4.8.3. Prevalence of lipase production

Sixty two (62%) isolates were found to produce extracellular protease while 38% isolates did not produce lipase as indicated by the zone of clearance around the colony on tributyrin agar plates. Clinical isolates (37.13%) were most prevalent in protease production than the environmental isolates (25.20%) (Figure 4.42). Among the total isolates 27% isolate showed the maximum zone of clearance (15-20mm) followed by 17% and 8% having (10-15) mm, (5-10)mm zone of clearance. Only 5% isolates showed extreme zone of clearance (<5 and >20mm) as shown in figure 4.43.

4.8.4. Prevalence of lecithinase production

Seventy three (73%) isolates were found to be extracellular lecithinase producer while 27% isolates did not produce lipase. Clinical isolates (45%) were most prevalent in protease production than the environmental isolates (28%) (Figure 4.42). Among the total isolates 32% isolate showed the maximum zone of clearance (10-15mm) followed by 16%, 11% and 8% having (15-20) mm, (5-10) mm zone of clearance. Only 6% isolates showed zone of clearance >20mm as shown in figure 4.43.

Table 4.3. Preliminary screening of *S. aureus* isolates.

	Sample screened	Preliminary confirmatory tests				Positive *S.aureus* isolates#
		Gram staining*	Catalase test	Coagulase test	Mannitol fermentation	
Clinical	527	296(56.16%)	271(51.42%)	264(50.09%)	264(50.09%)	264 (50.09%)
Environmental	345	192(55.65%)	186(53.91%)	180(52.17%)	180(52.17%)	180 (52.17%)
Total	872	488(55.96%)	457(52.40%)	444(50.91%)	444(50.91%)	444(50.91%)

*suspected positive isolates; # positive isolates

Table 4.5. Distance matrix showing relation between S. aureus clinical isolate (SaC53) and CA/environmental isolate (SaE31) with other *S. aureus, S. epidermidis* (Z26894.1|, X75944.1|, X75944.2| and *Pseudomonas aeruginosa* (KJ081977.1| and KJ081976.1|)strain.

SaC53		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
SaE31	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
D83356.1|	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
D83354.1|	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
D83353.1|	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
NR_115606.1|	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559244.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559243.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559242.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559241.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559240.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
HM559238.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.9986	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652903.1|	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		0.9986	0.9986	0.9986	0.9986	0.9986	0.9986	0.9986	0.9986	0.9986	1.7249	1.9890	1.9890	7.9357	7.9357
JN652902.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652901.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652898.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652897.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652894.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652893.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652890.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652889.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0000	1.4103	1.7249	1.7249	7.9357	7.9357
JN652888.1|	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		1.4103	1.7249	1.7249	7.9357	7.9357
Z26894.1|	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	3.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000	2.0000		0.9986	0.9986	7.8648	7.8648
X75944.1|	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	4.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	1.0000		0.0000	7.8648	7.8648
X75944.2|	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	4.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	3.0000	1.0000	0.0000		7.8648	7.8648
KJ081977.1|	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	79.0000	79.0000	79.0000		0.0000
KJ081976.1|	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	81.0000	79.0000	79.0000	79.0000	0.0000	

Table 4.8. Prevalence of *S. aureus* types in clinical and environmental isolates.

Origin of samples	No. of sample	No. (%) of *S. aureus* isolated	Phenotypical distribution		MRSA alone (%)		Total MRSA	MSSA alone (%)		Total MSSA
			Wild type	SCV	Wild type	SCV	Total	Wild type	SCV	Total
Clinical	527	264 (50.09%)	190(72%)	74(28%)	50(18.9%)	11(4.2%)	61(23%)	126(47.7%)	40(15.2%)	166 (63%)
Environmental	345	180 (52.17%)	121(67.2%)	59(32.8%)	39(21.6%)	06(3.3%)	45 (25%)	90(50%)	27(15%)	117 (65%)
Total	872	444 (50.92%)	311(70%)	133(30%)	89 (20%)	17(9.4%)	106 (23.9%)	216(48.6%)	90(15%)	283 (63.7%)

Table 4.11. The mean optical densities and standard deviations of the different biofilm phenotypes.

	Biofilm formation							
	Non-adherent		Weakly- adherent		Moderately-adherent		Strongly-adherent	
Parameters	**OD range**	**Mean OD ± SD**	**OD range**	**Mean OD ± SD**	**OD range**	**Mean OD ±SD**	**OD range**	**Mean OD ± SD**
37°C (24 h)	LTC	ND	0.123–0.159	0.133 ± 0.13	0.270–0.330	0.291 ± 0.15	LTC	ND
37 °C (48 h)	LTC	ND	0.141–0.176	0.153 ± 0.13	0.291–0.364	0.319 ± 0.25	0.442–0.569	0.498 ± 0.39
37 °C (72 h)	LTC	ND	0.136–0.211	0.188 ± 0.15	0.310–0.408	0.341 ± 0.07	0.481–0.879	0.678 ± 0.51
37 °C (96 h)	LTC	ND	0.121–0.183	0.148 ± 0.49	0.293–0.385	0.149 ± 0.19	0.445–0.731	0.571 ± 0.37

*LTC= lower than control; ND= not determined

Table 4.12. Sensitivity and specificity of phenotypic and genotypic method in biofilm formation.

Isolates	Method	Biofilm Classification	Positive phenotype	*ica* positive*	*ica* negative#	Sensitivity[1]%	Specificity[2]%
Clinical	Tube method	Adherent	228	219	9	96.1	91.6
		Non-adherent	36	3	33		
	CRA method	Adherent	164	129	35	78.6	88
		Non-adherent	100	12	88		
Environmental	Tube method	Adherent	134	124	10	92.5	95.7
		Non-adherent	46	2	44		
	CRA method	Adherent	111	88	23	79.3	81.2
		Non-adherent	69	13	56		

* ica positive: positive for *icaA* and *icaD*; # ica negative: negative for *icaA* and *icaD*

[1] Sensitivity = PCR +ve/phenotype +ve ; [2] Specificity = PCR –ve/ phenotype –ve

Table 4.13. The effects of surface and glucose on the biofilm formation of *S.aureus* isolates.

	No. of Isolates producing in-vitro biofilm								
	Glass(%)			Polystyrene(%)			PVC(%)		
Biofilm Phenotype	**Glucose(+)**	**Glucose(-)**	**Total**	**Glucose(+)**	**Glucose(-)**	**Total**	**Glucose(+)**	**Glucose(-)**	**Total**
NA	29	7	36	21	10	31	12	3	15
WA	27	5	32	36	8	44	35	0	35
MA	20	3	23	31	0	31	46	22	68
SA	22	2	24	35	4	39	47	19	66
Total adherence.	98	17	115	123	22	145	140	44	184
(%)	(22.08%)	(3.82%)	(25.90%)	(27.70%)	(4.95%)	(32.65%)	(31.53%)	(9.91%)	(41.44%)

*NA= non adherent; WA=weakly adherent; MA=moderately adherent; SA=strongly adherent.

Table 4.14. Prevalence of *icaABCD* genes and their biofilm formation pattern.

Isolates	**Biofilm classification**	**+ve phenotype**	***icaA***	***icaB***	***icaC***	***icaD***	***icaA+icaD***	**No *icaA/icaD***	***bap***
Clinical (n=264)	Strongly adherent	85(32%)	75(28.4%)	_	_	48(41.2%)	39(14.8%)	02(0.8%)	03(1.1%)
	Moderately adherent	66(25%)	49(18.6%)	_	_	35(42.4%)	18(6.8%)	17(6.4%)	02(0.8%)
	Weakly adherent	77(29.2%)	36(13.6%)	_	_	20(39%)	22(8.3%)	22(8.3%)	_
	Non adherent	36(13.6%)	2(0.8%)	_	_	01(2.8%)	0(0%)	33(12.5%)	_
	Total	228(86.4%)	162(61.4%)			104(39.4%)	79(29.9%)	74(28%)	05(1.9%)
Environmental (n=180)	Strongly adherent	44(24.4%)	36(20%)	_	_	38(21.1%)	12(6.7%)	12(6.7%)	07(3.9%)
	Moderately adherent	56(36.7%)	44(24.4%)	_	_	47(26.1%)	11(6.1%)	9(5%)	02(1.1%)
	Weakly adherent	34(18.9%)	18(10%)	_	_	18(10%)	6(3.3%)	6(3.3%)	_
	Non adherent	46(25.6%)	2(1.1%)	_	_	2(1.1%)	0(0%)	41(22.7%)	_
	Total	134(74.4%)	100(55.6%)			105(58.3%)	29(16.1%)	68(37.8%)	09(5.0%)

Table 4.15. Prevalence of *hla* and *hlb* gene and their haemolysis pattern.

Isolates	**Haemolysis classification**	**Positive phenotype**	***hla* gene**	***hlb* gene**	***hla*+*hlb* gene**	**No *hla*/*hlb* gene**
Clinical(n=264)	Beta-haemolysis	134(50.76%)	88(33.3%)	95(36%)	70(26.5%)	21(8.0%)
	Alpha-haemolysis	56(21.21%)	33(12.5%)	39(14.8%)	24(9.0%)	8(3.0%)
	Gamma-haemolysis	74(28.03%)	_	_	_	_
	Total	190(72%)	121(46%)	134(50.8%)	94(35.5%)	29(11%)
Environmental(n=180)	Beta-haemolysis	101(56.11%)	62(34.4%)	70(38.9%)	46(25.6%)	15(8.3%)
	Alpha-haemolysis	20(11.11%)	17(9.4%)	17(9.4%)	16(8.9%)	02(1.1%)
	Gamma-haemolysis	59(32.8%)	_	_	_	_
	Total	121(67.2%)	79(44%)	87(48.3%)	62(34.4%)	17(9.4%)

Table 4.16. Prevalence of *sea* and *sed* gene in clinical and CA/environmental isolates.

Isolates	***sea*(%)**	***seb*(%)**	***sec*(%)**	***sed* (%)**	***sea*+*sed*(%)**	**No *sea*/*sed***
Clinical (n=264)	155(58.7%)	-	-	134(50.7%)	117(44%)	41(15.5%)
Environmental (n=180)	133(74%)	-	-	66(36.7%)	40(22.2%)	50(27.8%)
Total (n=444)	288(65%)	-	-	200(45%)	157(35.4%)	91(20.5%)

Results

Table 4.1. Prevalence of *S. aureus* from clinical sources.

Samples parameters	Samples screened	Frequency
Age(years)		
0-5	68	41(60.3%)
6-20	112	56(50%)
21-40	145	49(33.8%)
41-60	123	48(39%)
80 & above	79	70(88.6%)
Sex		
Male	321	152(47.4%)
Female	206	112(54.4%)
Locality		
Urban	156	117(75%)
Rural	371	147(39.6%)
Sample source		
Pus	139	72(51.8%)
Sputum	43	16(37.2%)
Urine	61	44(72.1%)
Throat swab	89	28(31.5%)
Nasal swab	61	48(78.7%)
Oral swab	103	48(46.7%)
Blood	31	8(25.8%)
Total	527	264(50.09%)

Results

Table 4.2. Prevalence of *S. aureus* from environmental sources.

Sample source	Sampling location			Total sample screened	Frequency
	Cachar	Karimganj	Hailakandi		
Raw milk	24	31	21	76	49 (64.5%)
Packaged milk	11	9	19	39	24 (61.5%)
Sweets					
Milk barfi	11	5	3		
Kalakad	4	7	3	42	15 (35.7%)
Rasgulla	2	5	2		
Dairy equipments	7	3	5		
Milking cane	4	2	7	44	13 (29.5%)
Milk jug	5	6	5		
Measuring jug					
Meat surface					
Cutting knife	5	2	8		
Containers	3	2	5	41	11 (26.8%)
Cutting surface	7	4	5		
Paneer	18	8	5	31	20 (64.5%)
Cheese	3	7	7	17	9 (52.9%)
Milk handlers hand	6	5	4	15	7 (46.7%)
Normal skin flora	15	10	15	40	32 (80%)
Total	125	106	114	345	180 (52.17%)

Results

Table 4.4. Morphological and Biochemical characterization of the isolates.

Morphological characterization		**Biochemical characterization**	
Test	**Reaction**	**Test**	**Reaction**
Gram reaction	++	Catalase	++
Colony shape	Circular +++	Coagulase	+++
Cell shape	Coccus +++	Oxidase	–
Cell diameter	1+, 2+	Indole Production	+
Elevation	Convex+++	Nitrate Reduction	+++
Texture	Rough++	Citrate	+
Appearance	Shiny++, opaque+	Methyl Red	+++
Pigmentation	Golden yellow+++	Voges- Proskauer	+++
Motility	–	Alkaline phosphotase	+
Spores	–	Urease	+++
		Acid from sugar	
		a) Glucose	$+++^{1g\ 1a}$
		(b) Mannitol	$+++^{2g\ 2a}$
		c) Maltose	$+++^{3g\ 3a}$
		(d) Lactose	$+^{3g\ 1a}$
		e) Ribose	$+^{1a}$
		(f) Sucrose	$++^{3g\ 3a}$
		(g) fructose	$+++^{4a}$

1+ = 4-6mm, 70% positive; 2+ = 1-3mm 30% positive.
+++ = 100% positive; ++ = 80% - 90% positive; + = 60% - 70% positive; – = 100% negative.
^{1g} = 60% gas production; ^{2g} = 40% gas production; ^{3g} = 20% gas production.
^{1a} = 40% acid production; ^{2a} = 60% acid production; ^{3a} = 80% acid production; ^{4a} = 100% acid production.

Results

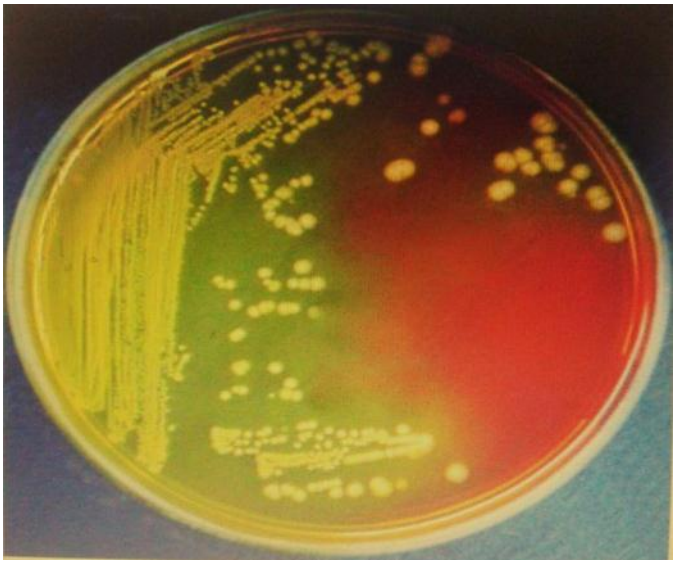

Figure 4.1. ***Staphylococcus aureus*** **grown on Mannitol Salt Agar (MSA)** at 37°C for 48 hours, with yellow halo around colony because of fermentation of mannitol.

Figure 4.2. ***S. aureus*** **small colony variants (SCV) grown on Mannitol Salt Agar** (MSA) at 37°C for 48 hours reveals comparatively small colonies.

Results

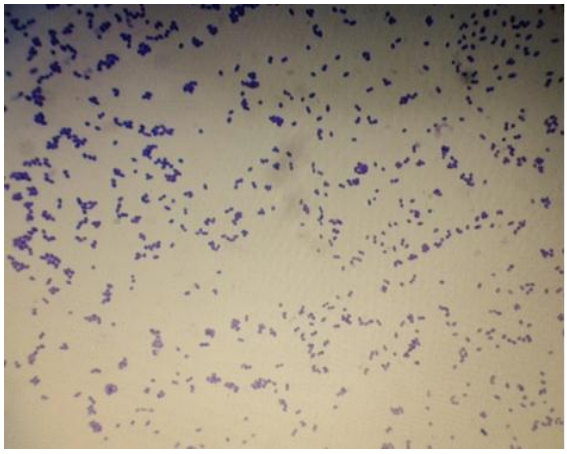

Figure 4.3. Gram reaction of *S. aureus*

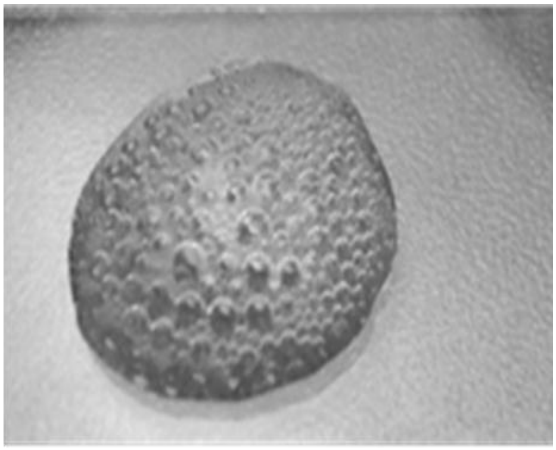

Figure 4.4. Catalase reaction of *S. aureus* .

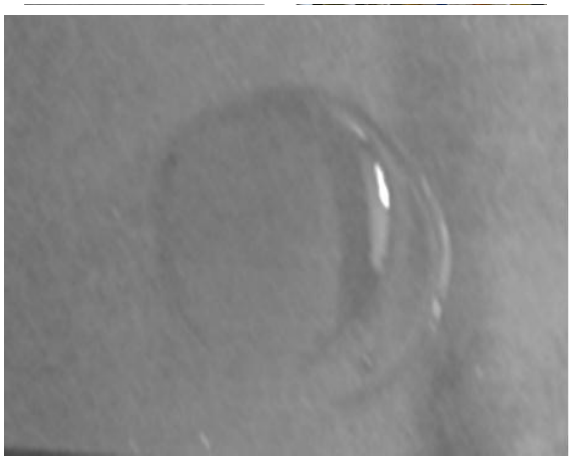

Figure 4.5. clot formation for coagulase positive test of *S. aureus*.

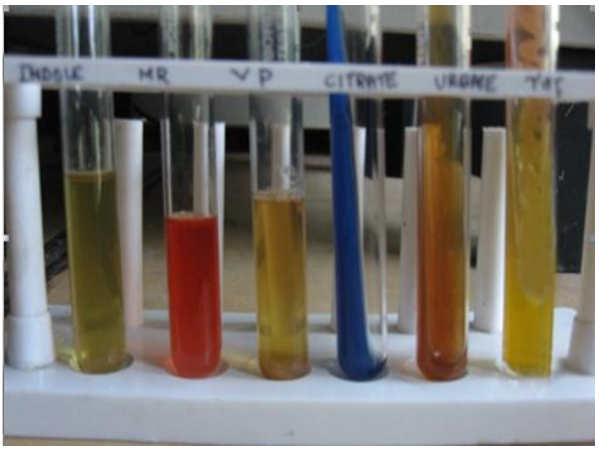

Figure 4.6. Biochemical characterization :-

Indole(-ve), MR(+ve),

VP(-ve), Citrate(+ve), Urease(+ve),

TSI(Yellow in slant).

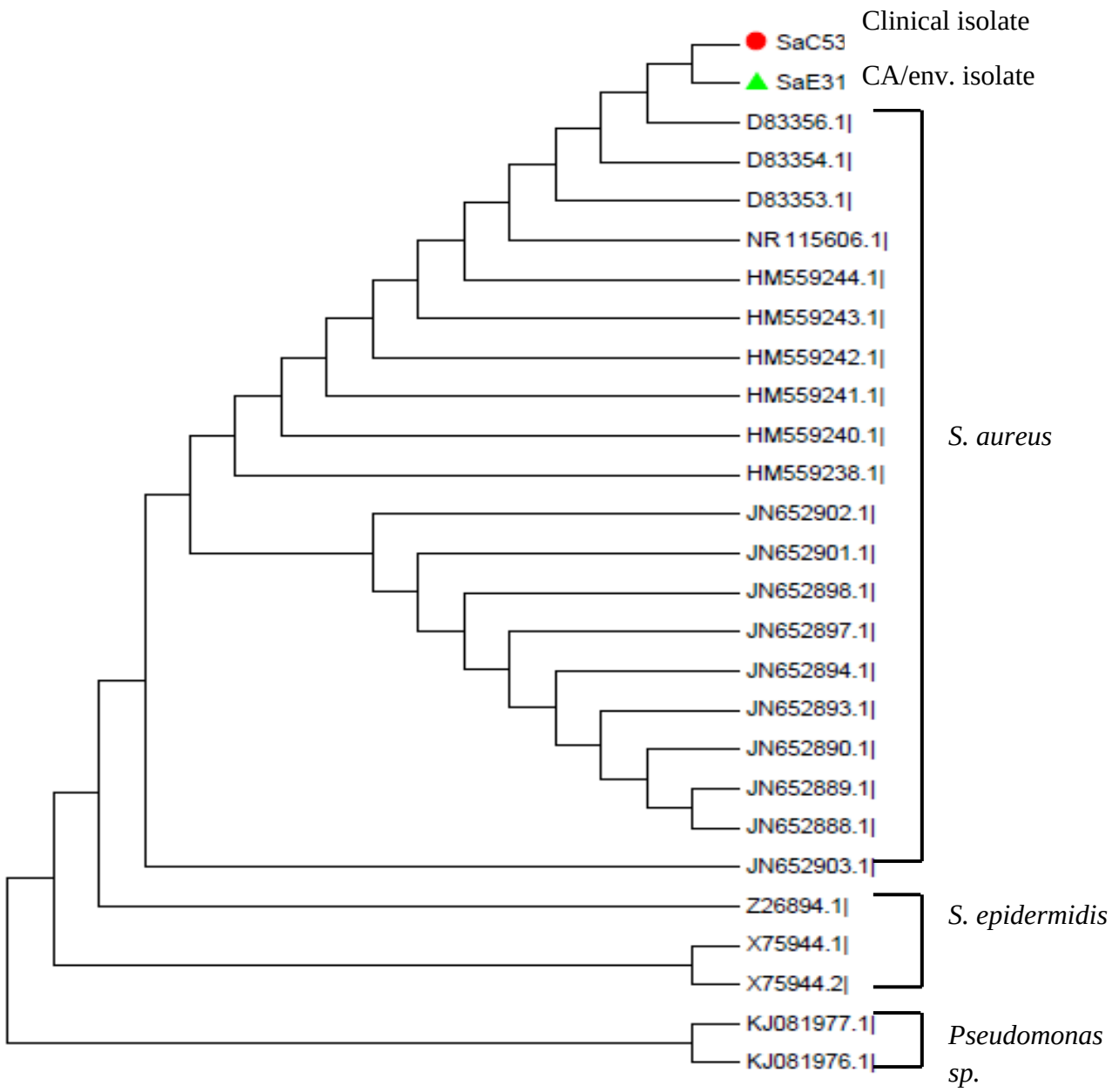

Figure 4.7. Phylogenetic tree showing relation between *S. aureus* clinical isolates (SaC53) and CA/Environmental isolate (SaE31) with other *S.aureus, S.epidermidis* and *Pseudomonas.sp.* *The* phylogenetic tree was constructed based on the nucleotide sequences of 16SrRNA gene MEGA-5 programme with the bootstrap test of phylogeny in neighbour-joining method using the 1000 replica of data set (Tamura et al. 2007). The genetic distance is the number of base substitutions per site. Isolates having same sequences are not repeated except when the isolation location is different and epidemiologically important.

Results

Table 4.6. Percentage of clinical isolates sensitive/resistant to different antibiotics.

Antibiotics*	**Clinical isolates**		
	S_n(%)	**R_n(%)**	**I_n(%)**
Vancomycin (10µg)	100	0	0
Linzoid (30 µg)	100	0	0
Oxacillin (5µg)	63	23	14
Amikacin (30µg)	45	25	30
Ciprofloxacin (5µg)	54	20	23
Gentamicin (10µg)	10	69	21
Ceftriaxone (30µg)	43	46	23
Erythromycin (15µg)	38	27	35
Cefuroxime (30µg)	43	33	23
Tetracycline (30µg)	25	50	25
Penicillin (10IU)	0	100	___
Ampicillin (10µg)	0	100	___

S_n = sensitive isolates; R_n= resistant isolates; I_n= intermediate isolates.

*The concentration of antibiotics are given in parenthesis.

____ no values for diameter zones of inhibition according to NCCLS.

Results

Table 4.7. Percentage of environmental isolates sensitive/resistant to different antibiotics.

Antibiotics*	**Environmental isolates**		
	S_n(%)	**R_n(%)**	**I_n(%)**
Vancomycin (10µg)	100	0	0
Linzoid (30 µg)	100	0	0
Oxacillin (5µg)	65	25	10
Amikacin (30µg)	45	15	30
Ciprofloxacin (5µg)	51	15	34
Gentamicin (10µg)	21	56	23
Ceftriaxone (30µg)	51	29	20
Erythromycin (15µg)	44	20	36
Cefuroxime (30µg)	54	23	23
Tetracycline (30µg)	28	41	31
Penicillin (10IU)	4	96	____
Ampicillin (10µg)	0	100	____

S_n = sensitive isolates; R_n= resistant isolates; I_n= intermediate isolates.

*The concentration of antibiotics are given in parenthesis.

____ no values for diameter zones of inhibition according to NCCLS.

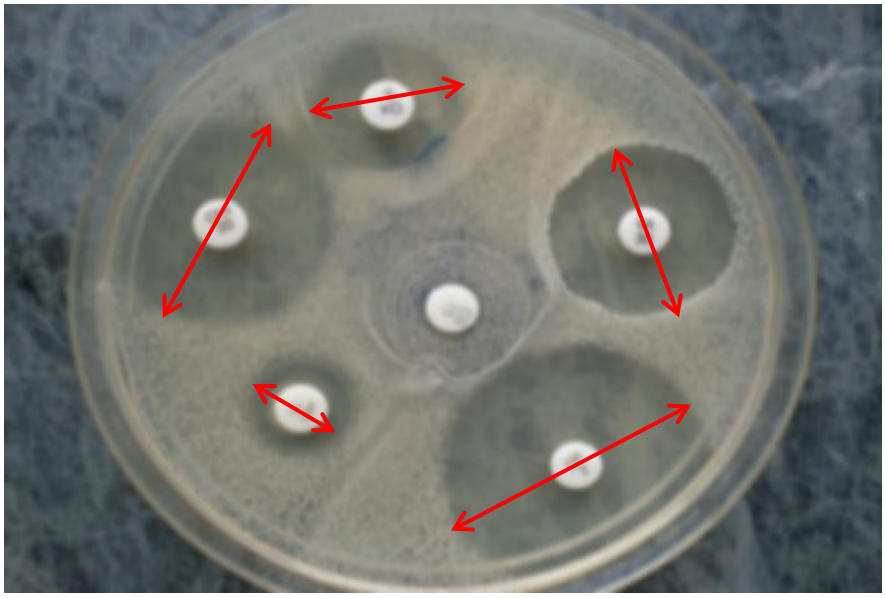

Figure 4.8. Antibiotic susceptibility test of *S. aureus* clinical isolate C1647 bydisc diffusion method showing zones of susceptibility against different antibiotics : vancomycin (10µg) oxacillin (5µg) cefuroxime (30µg) and amikacin (30µg) and penicillin (10IU).

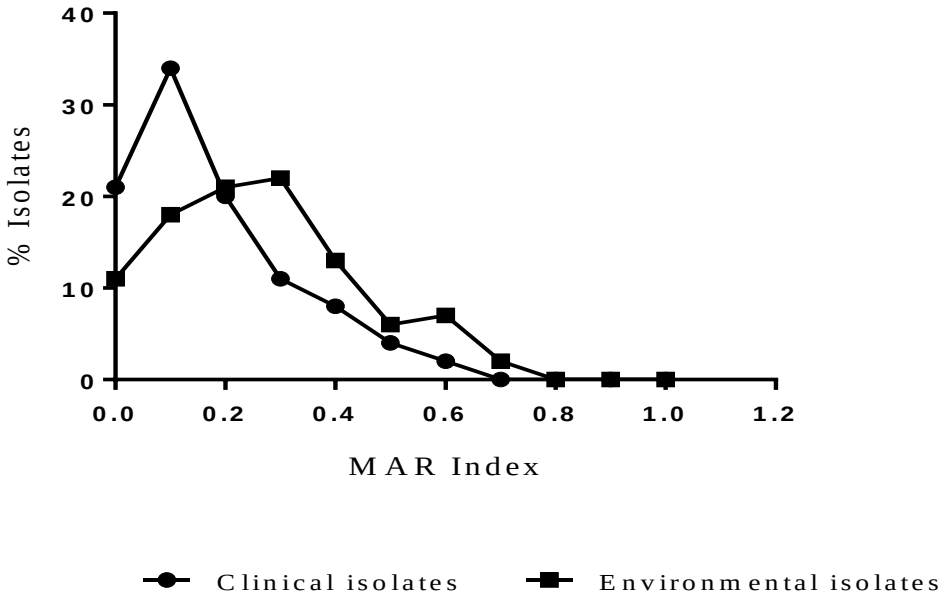

Figure 4.9. Comparative MAR index of both clinical and environmental *S. aureus* isolates against a range of antibiotics.

Table 4.9. Comparative biofilm formation pattern of clinical and environmental isolates by CRA method and Tube method the isolates.

No. of isolates	Biofilm formation	Screening methods	
		CRA	**TM**
Clinical isolates(n=264)	Strong	55(20.8%)	85(32.2%)
	Moderate	60(22.7%)	66(25%)
	Weak	49(18.6%)	77(29.2%)
	Non producer	100(37.9%)	36(13.6%)
Environmental isolates(n=180)	Strong	30(16.7%)	44(24.4%)
	Moderate	53(29.4%)	56(36.7%)
	Weak	28(15.6%)	34(18.9%)
	Non producer	69(38.3%)	46(25.6%)

Table 4.10. Prevalence of biofilm formation by Tube method in all *S. aureus* isolates.

Biofilm formation (OD_{620nm})	**No. of isolates (n=444)**
Strongly adherent (>= 0.440)	129 (29.05%)
Moderately adherent (>=0.220 to <=0.440)	122 (27.5%)
Weakly adherent (>0.110=<0.220)	111 (25.2%)
Non- adherent(<0.110)	82 (18.5%)

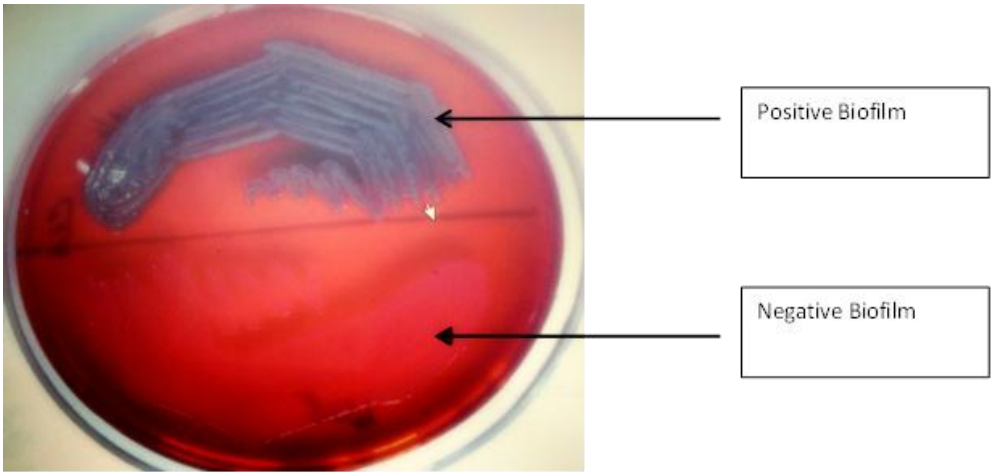

Figure 4.10. *In-vitro* biofilm formation by congo red agar (CRA) method. Black colonies confirms positive biofilm while pink colonies infers negative biofilm.

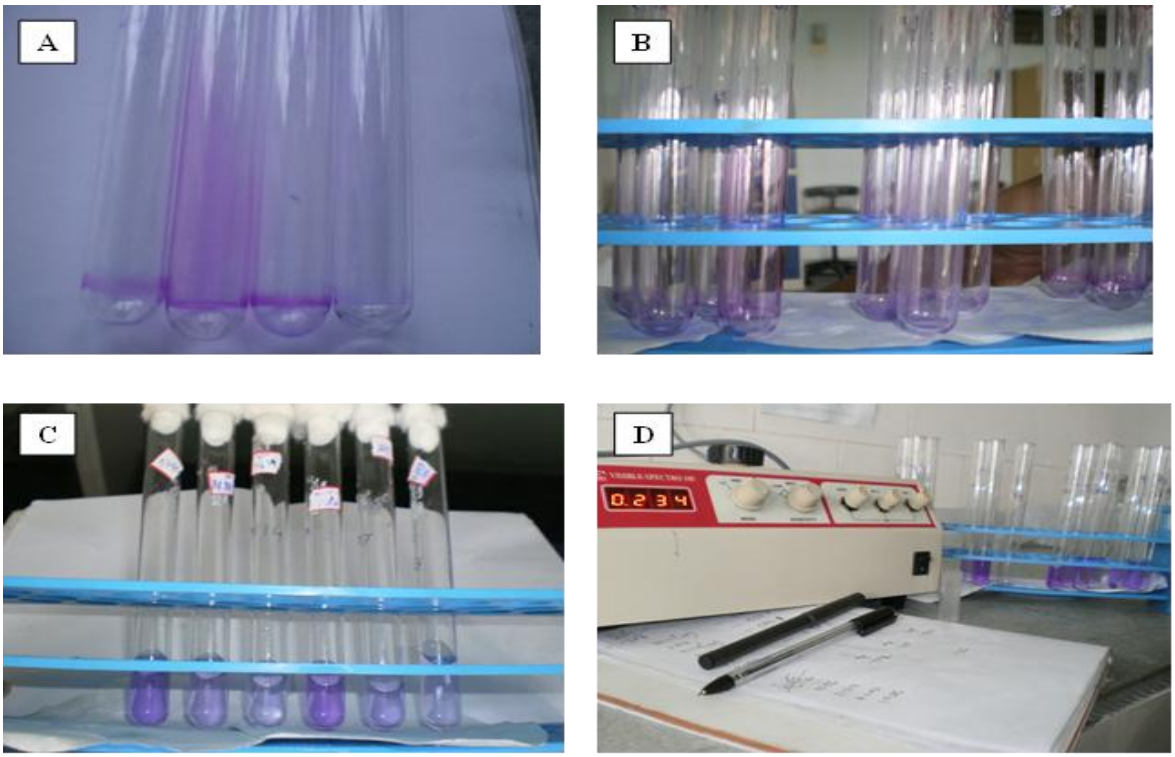

Figure 4.11. *In-vitro* biofilm formation by *S. aureus* isolates by tube method (TM). (A), (B) biofilm production in tubes. (C) biofilms dissolved in 95% ethanol (D) OD measured at 620nm in spectrophotometer (Systronics, 105N).

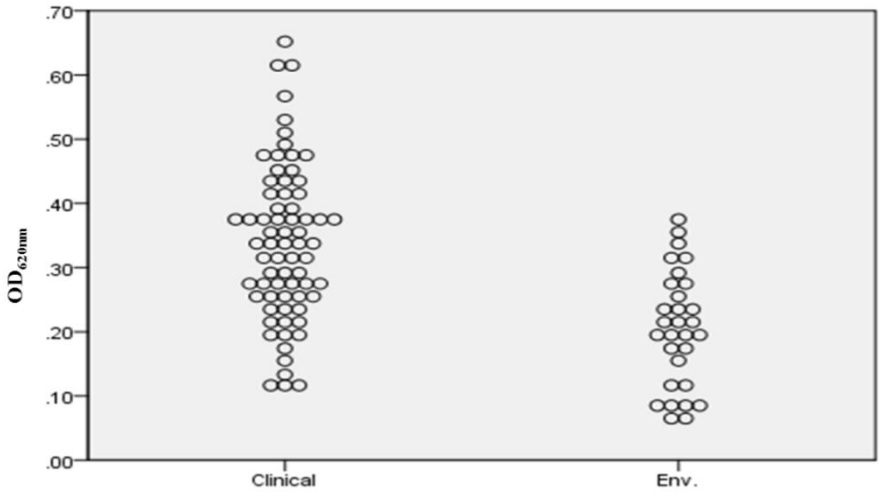

Figure 4.12. Comparative adherence values of clinical (n=70) and environmental (n=30) isolates of *S. aureus* by quantitative biofilm estimation method at 620nm optical density.

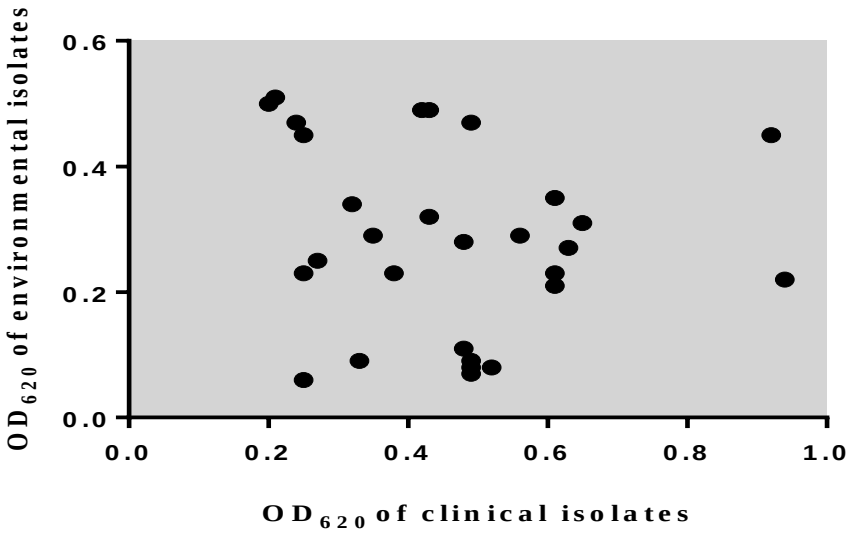

Figure 4.13. Correlation between adherence values of selected clinical and environmental isolates of *S. aureus* by quantitative biofilm estimation method at 620nm optical density.

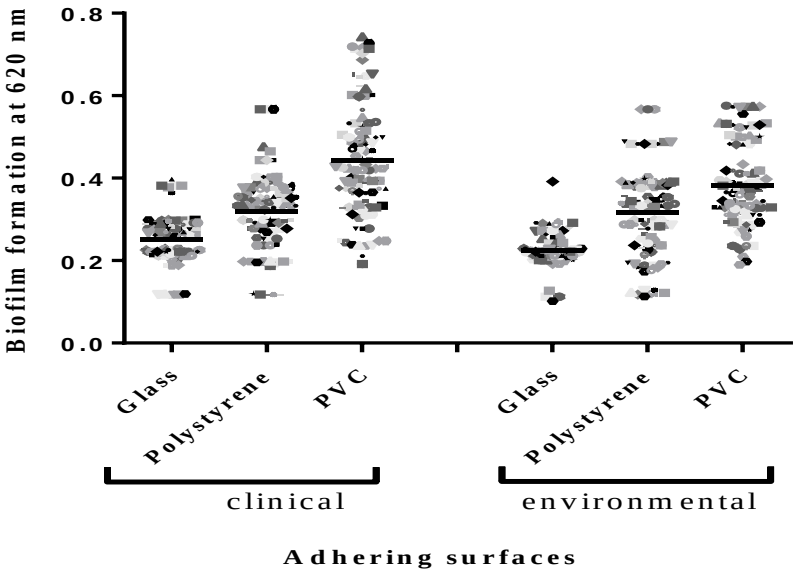

Figure 4.14. Comparative biofilm formation on different surfaces (glass, polystyrene and PVC) in clinical and environmental isolates of *S. aureus* by tube method at 620nm optical density.

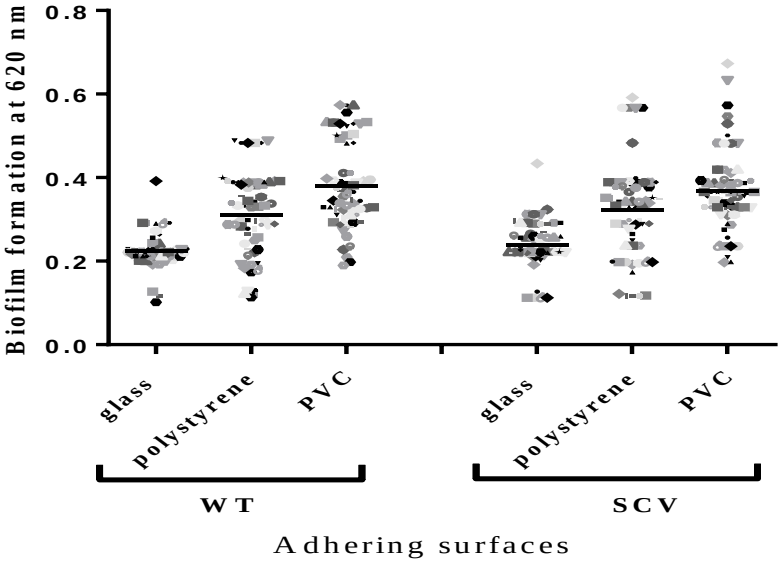

Figure 4.15. Comparative biofilm formation on different surfaces (glass, polystyrene and PVC) by WT and SCV isolates of *S. aureus* at 620nm optical density.

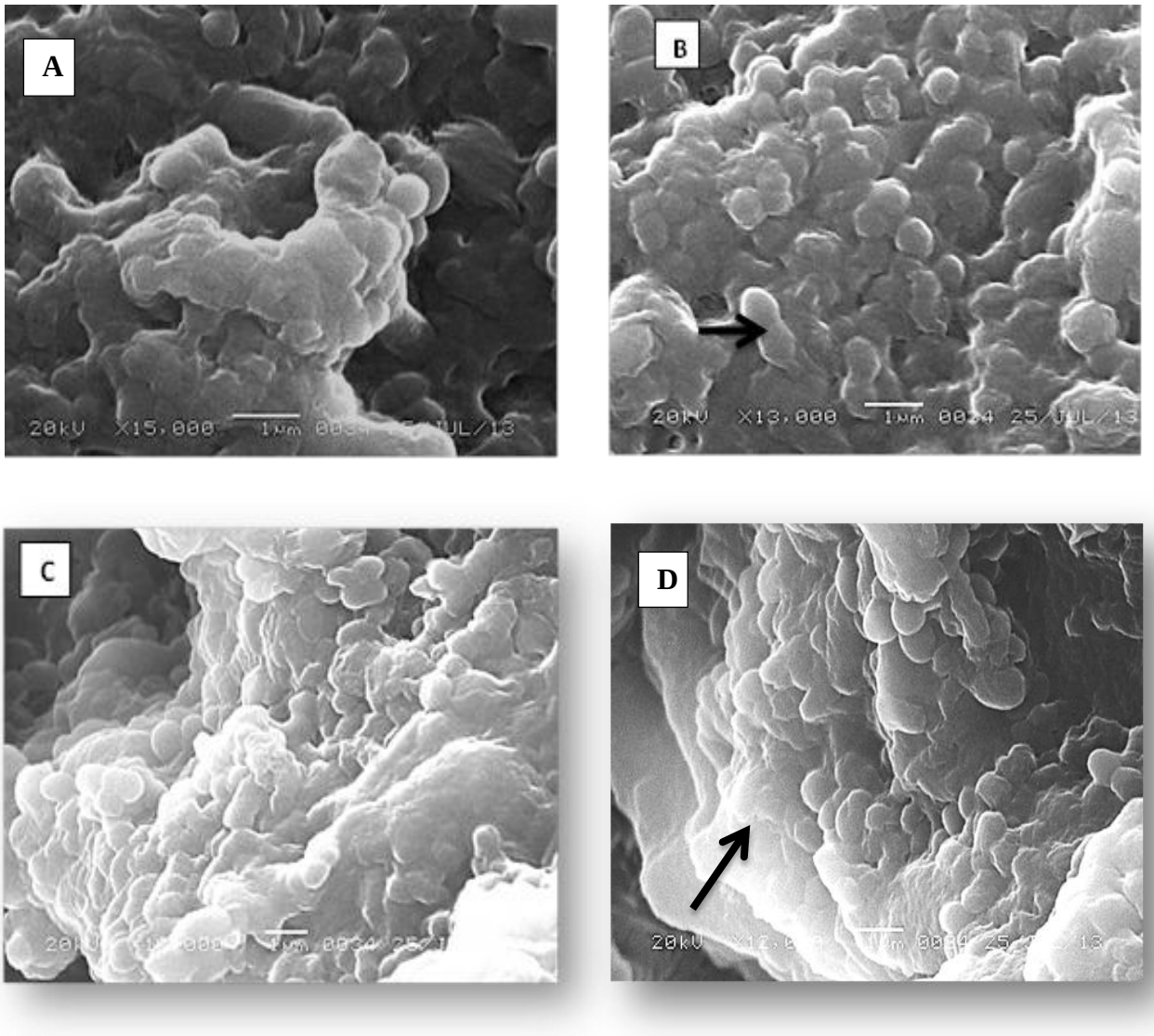

Figure 4.16. SEM micrograph of *In-vitro* biofilm formation on different surfaces- Glass (A,B) , PVC (C), Polystyrene (D). The multiplying cells embedded in common biofilm matrix is evident (A, B). Also, amount of biofilm formed was higher on PVC (C) as compared to glass(A,B) and polystyrene (D) as visually observed.

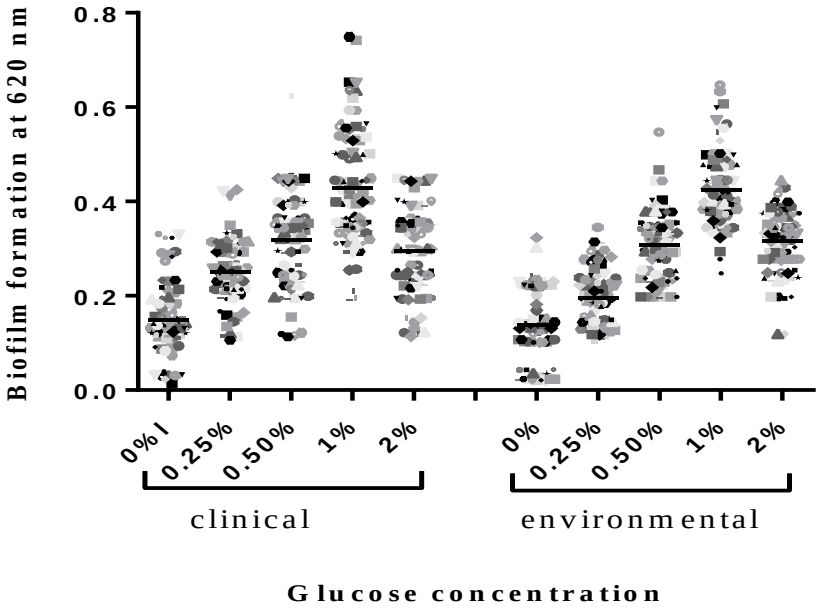

Figure 4.17. Effect of different glucose conc. (0%, 0.25%, 0.50%, 1%, 2%) on biofilm formation by *S. aureus* isolates of both clinical and environmental origin.

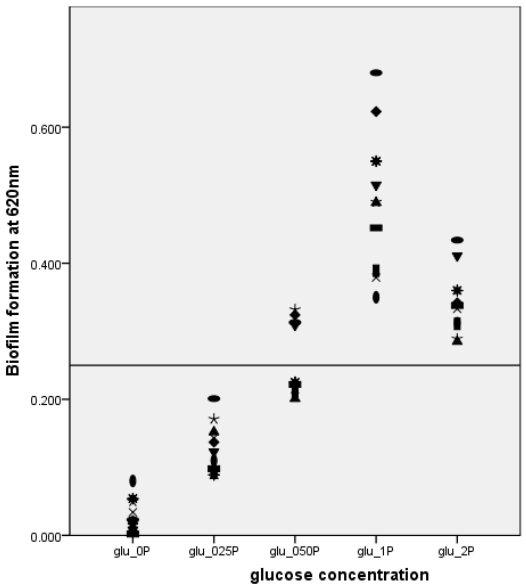

Figure 4.18. Effect of glucose stress on biofilm formation by selected SCV type *S. aureus* isolates of both clinical and environmental origin.

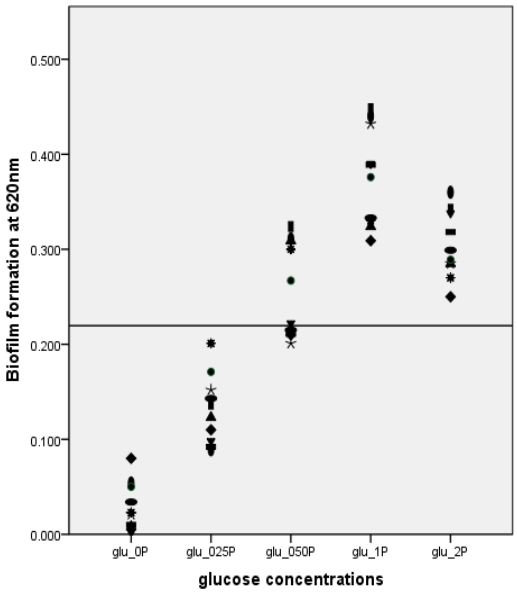

Figure 4.19. Effect of glucose stress on biofilm formation by selected WT *S. aureus* isolates of both clinical and environmental origin.

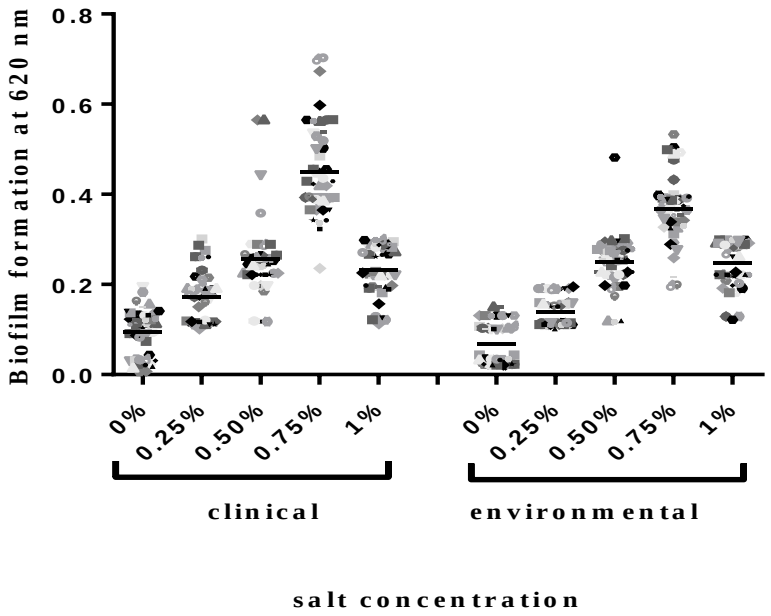

Figure 4.20. Effect of different salt conc. (0%, 0.25%, 0.50%, 0.75%, 1% and 2%) on biofilm formation by *S. aureus* isolates of both clinical and environmental origin.

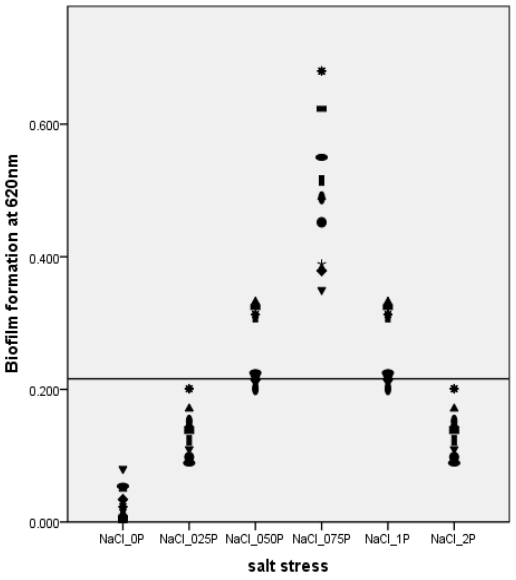

Figure 4.22. Effect of salt stress on biofilm formation by selected WT *S. aureus* isolates of both clinical and environmental origin.

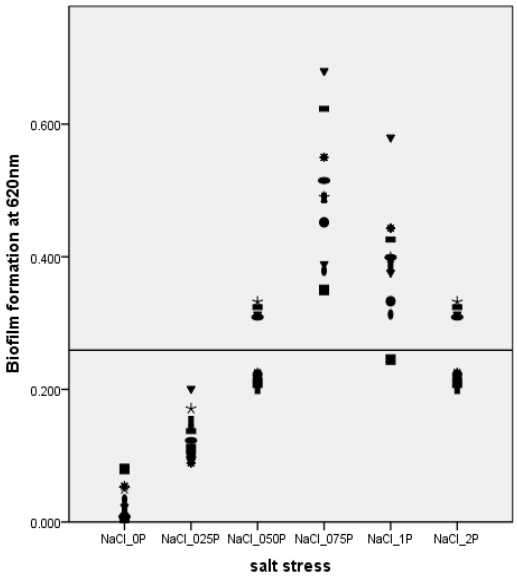

Figure 4.21. Effect of salt stress on biofilm formation by selected SCV *S. aureus* isolates of both clinical and environmental origin.

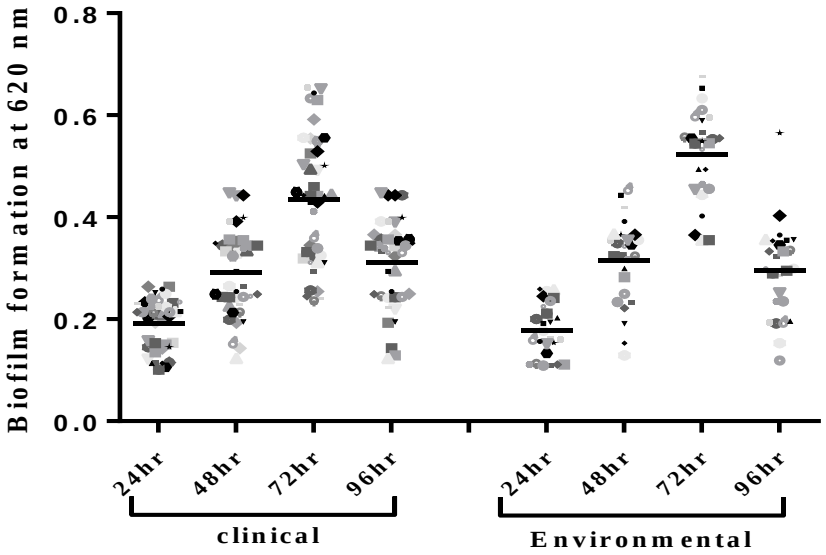

Figure 4.23. Effect of different incubation period (24h, 48h, 72h and 96h) on biofilm formation by clinical and environmental *S. aureus* isolates. Maximum biofilm formation is evident at 72hrs of incubation.

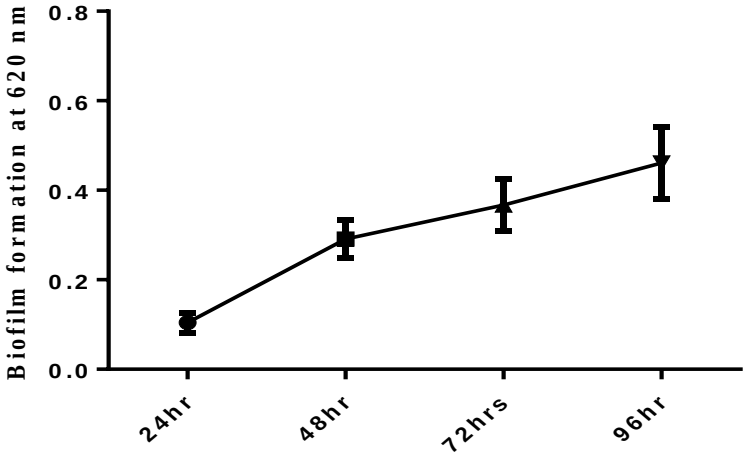

Figure 4.24. Effect of different incubation period (24h, 48h, 72h and 96h) on biofilm formation by selected *S. aureus* isolates showing maximum biofilm formation at 96hrs of incubation.

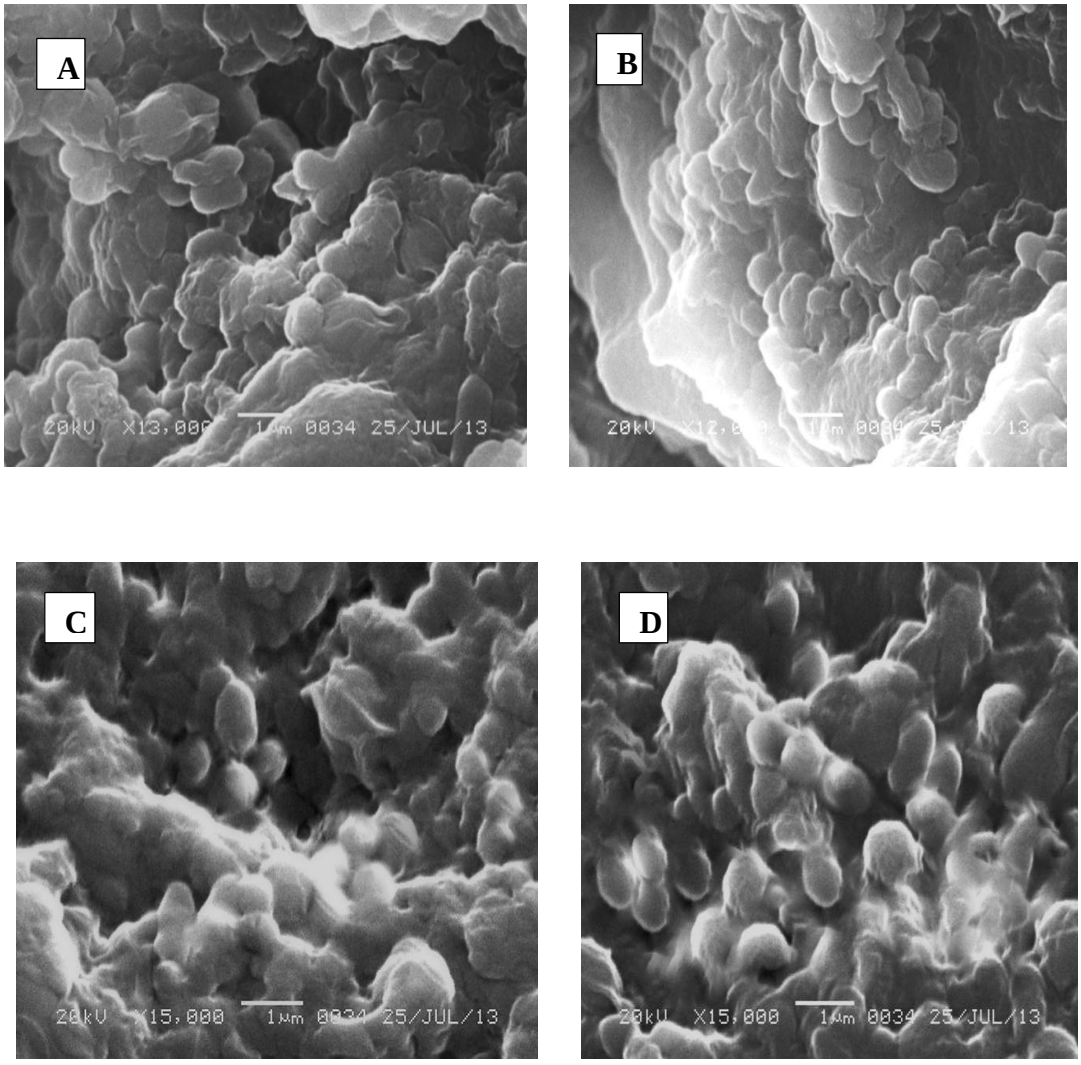

Figure 4.25. SEM micrograph of *In-vitro* biofilm formation at different incubation periods- 24hrs/1day (A), 48hrs/2days (B), 72hrs/3days (C) 96hrs/4days (D). A dense biofilm matrix is visible at 72hrs/3days incubation and the *S. aureus* cells are overlapped by biofilm matrix is observed.

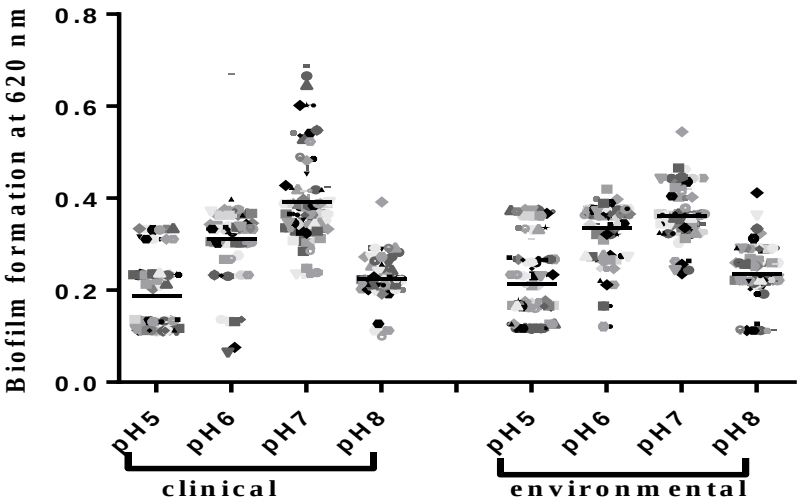

Figure 4.26. Effect of pH stress on biofilm formation by both clinical and environmental *S. aureus* isolates.

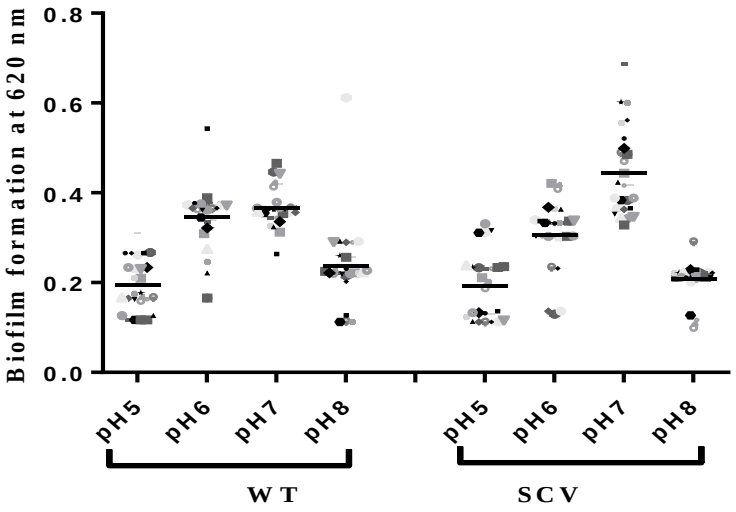

Figure 4.27. Effect of pH stress on biofilm formation by both WT and SCV phenotype of *S. aureus* isolates.

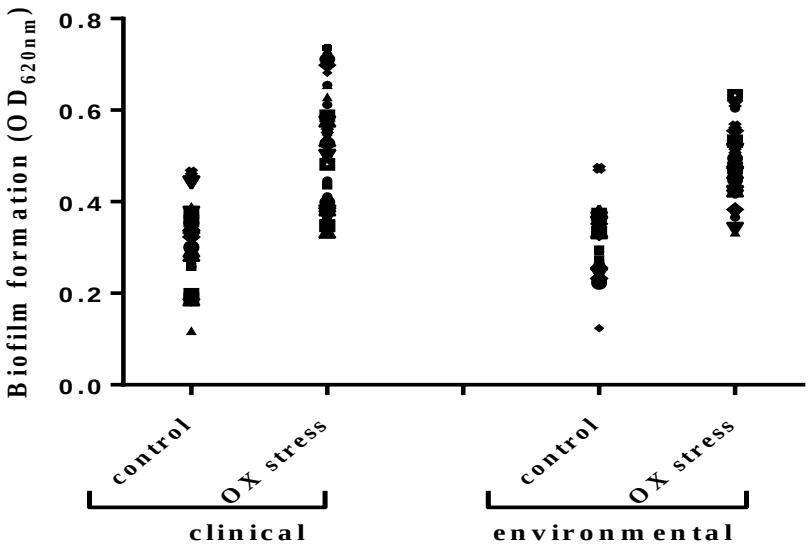

Figure 4.28a. Effect of antibiotic (OX=oxacillin) on biofilm formation by clinical and environmental isolates of *S. aureus* by quantitative biofilm estimation method at 620nm optical density.

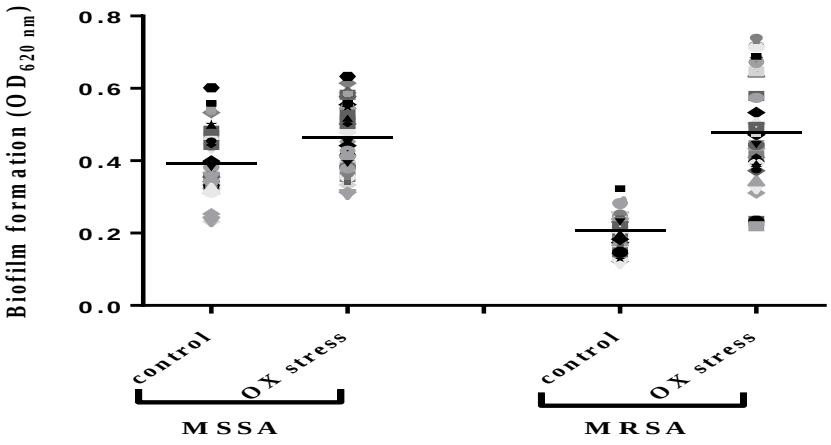

Figure 4.28b. Effect of antibiotic (OX=oxacillin) on biofilm formation by MSSA and MRSA by quantitative biofilm estimation method at 620nm optical density.

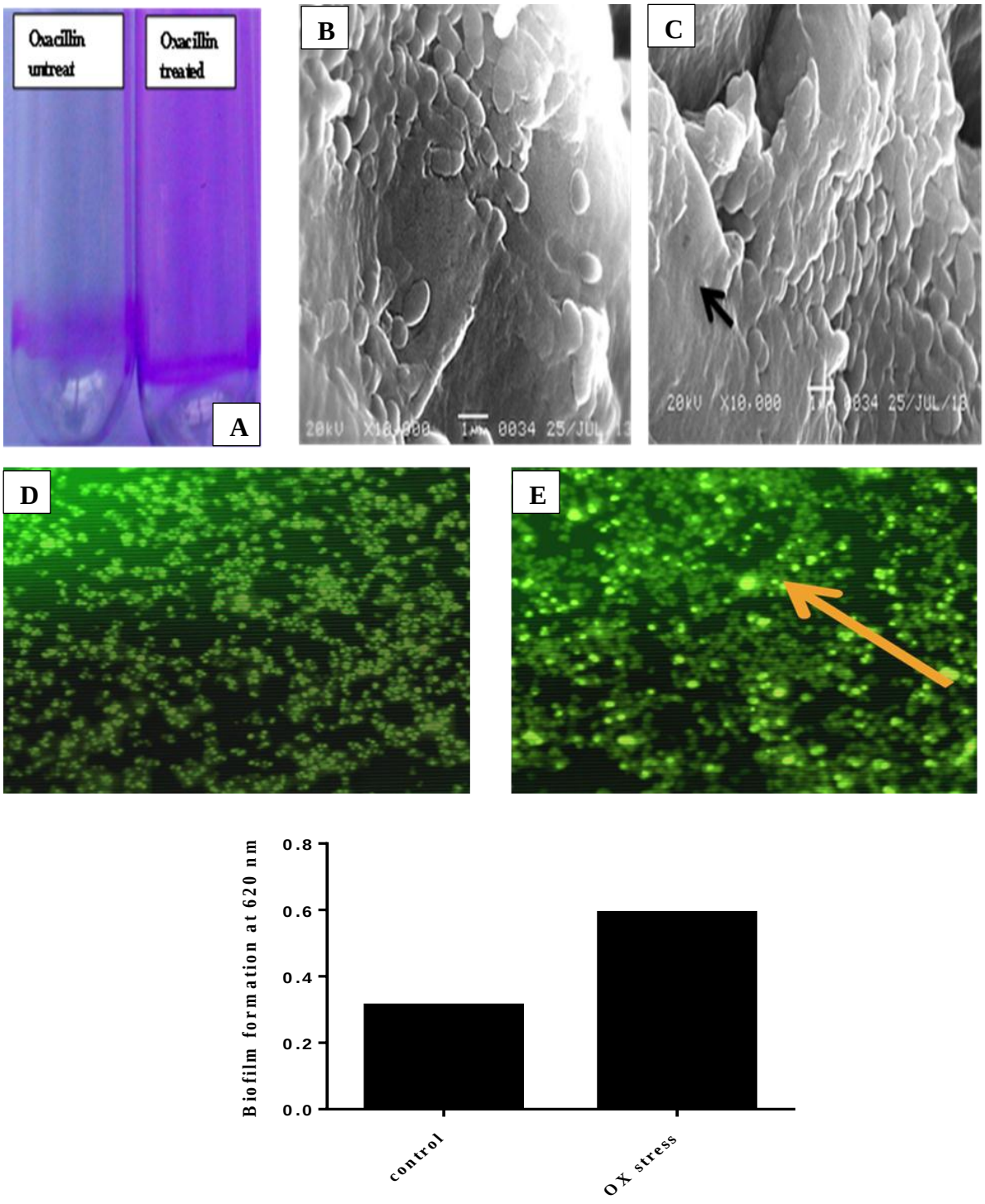

Figure 4.29. (A) *In-vitro* biofilm formation by *S. aureus* isolate (C4075) in tubes. (B) SEM micrograph of biofilm production (control) and (C) under antibiotic stress (Oxacillin). (D) CLSM micrograph of biofilm production (control) and (E) under antibiotic stress (Oxacillin). Graph showing the effect of oxacillin in biofilm formation by *S. aureus* isolate (C4075).

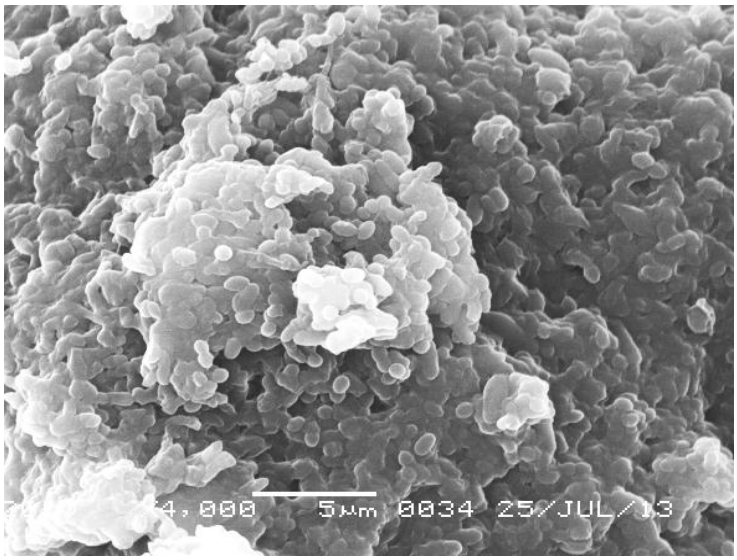

Figure 4.30. SEM micrograph of clinical *S. aureus* isolate (C4075).

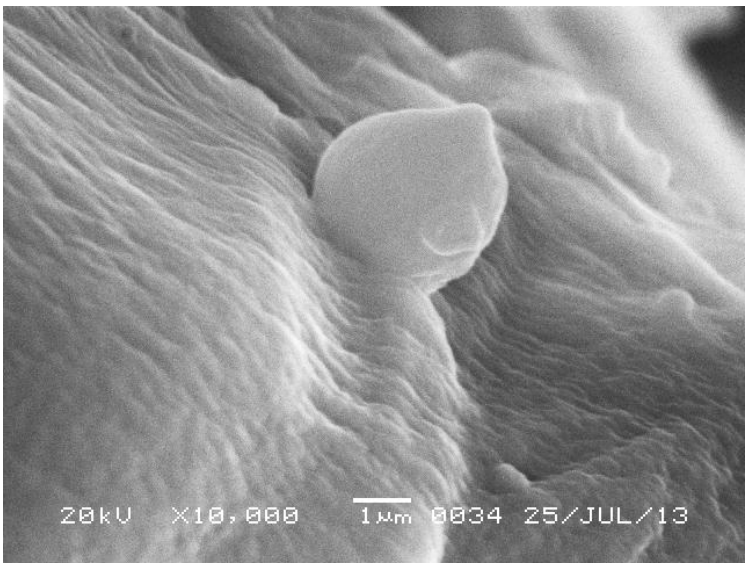

Figure 4.31. SEM micrograph of a single SCV cell of *S. aureus* isolate (SaC64).

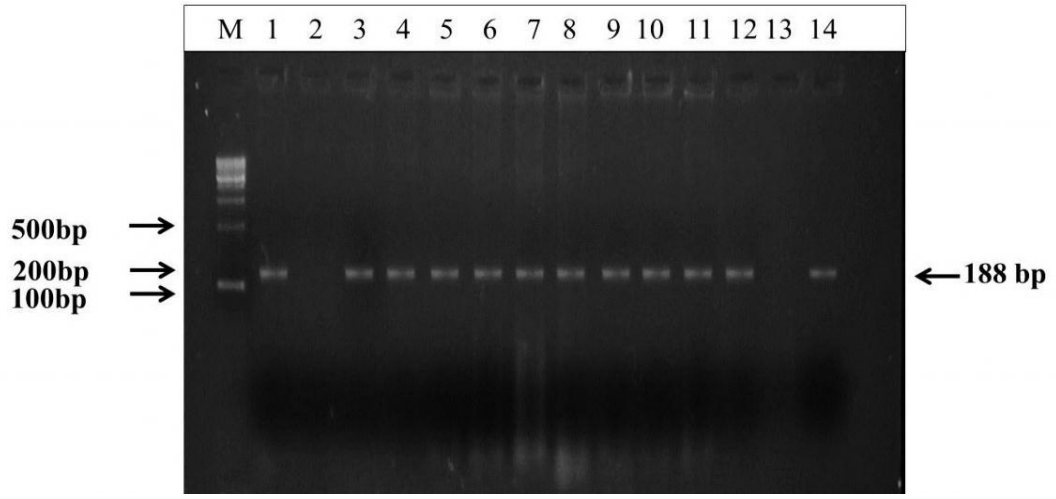

Figure 4.32. Molecular detection of *icaA* gene. lane1= positive control, lane2= negative control, lane3-14=188bp *icaA* gene (isolates CP99, CU11, CU33, CP301, CBM, CNS55, EP19, ENS11, ER 11, ER01 in lanes respectively) M= marker.

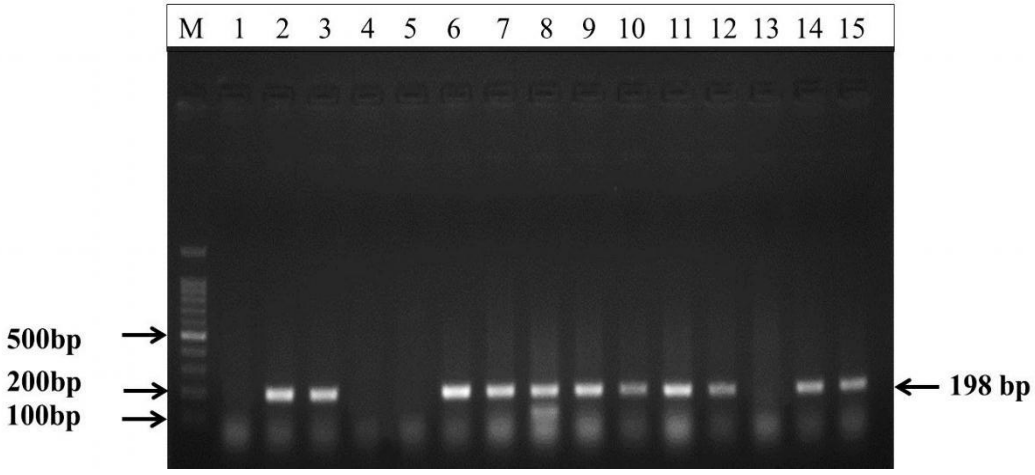

Figure 4.33. Molecular detection of *icaD* gene. lane1= negative control, lane2= positive control, lane3-14= 188bp *icaD* gene (CP11, CU11, CNS15, CNS81, CS91, CS22, CTS67, COS103, EE05, EK05, EHH31, EC09 in lanes respectively), M= marker.

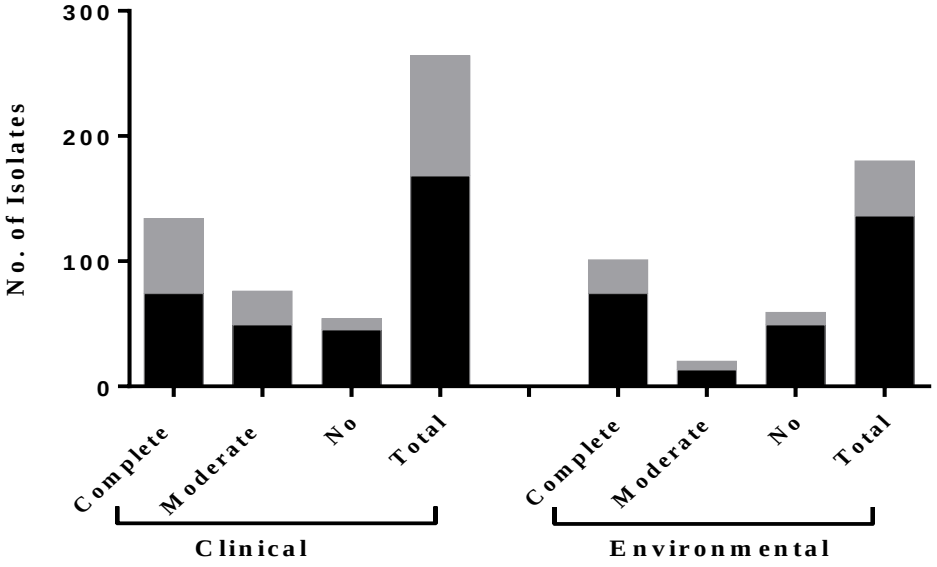

Figure 4.34. Comparative haemolysis pattern exhibited by MRSA and MSSA biotypes of clinical and environmental isolates of *S. aureus* . =MSSA =MRSA.

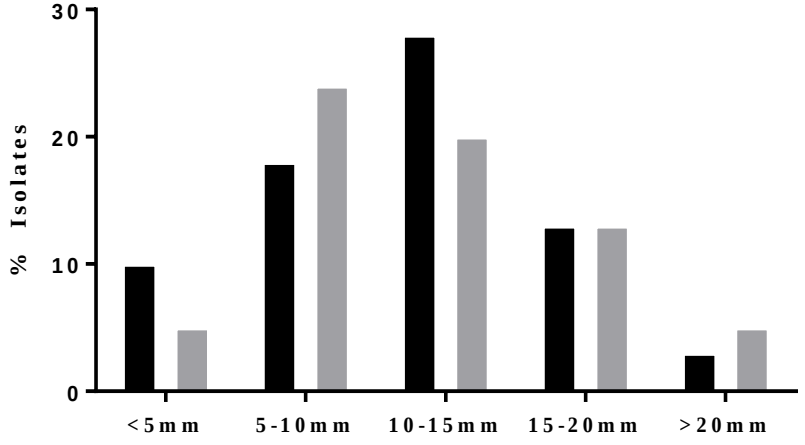

Figure 4.35. Comparative haemolysis zone sizes displayed by both clinical and environmental isolates of environmental isolates of *S. aureus* . =Clinical isolates = Environmental isolates.

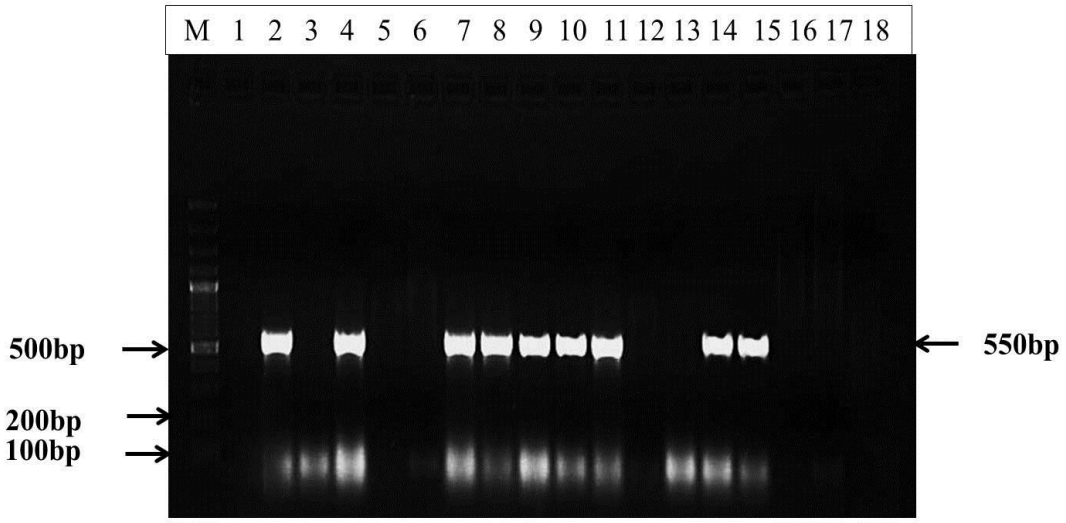

Figure 4.36. Molecular detection of *hla* gene. lane1= negative control, lane2= positive control, lane3-15= 550bp *hla* gene (isolates ES09, EP05, ENS22, E46, ER09, CU11, CPM1, CP22, CNS23, C35, C4071, CS87, CU31 respectively in lanes), M= marker.

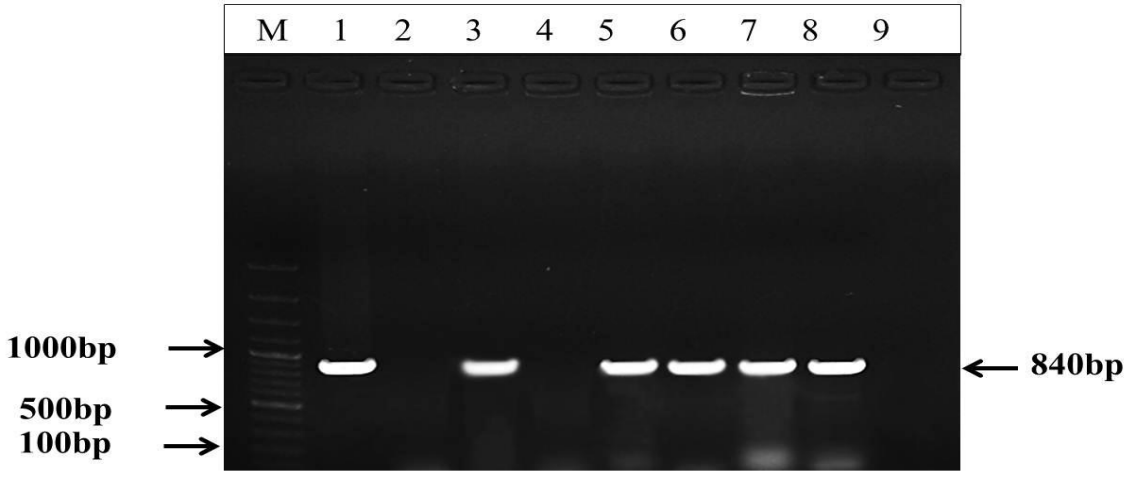

Figure 4.37. Molecular detection of *hlb* gene. lane1= positive control, lane2= negative control, lane3-9=840bp *hlb* gene (isolate CE71, CN02, CU11, EP03, EN31, ER09, E101 respectively in lanes), M= marker.

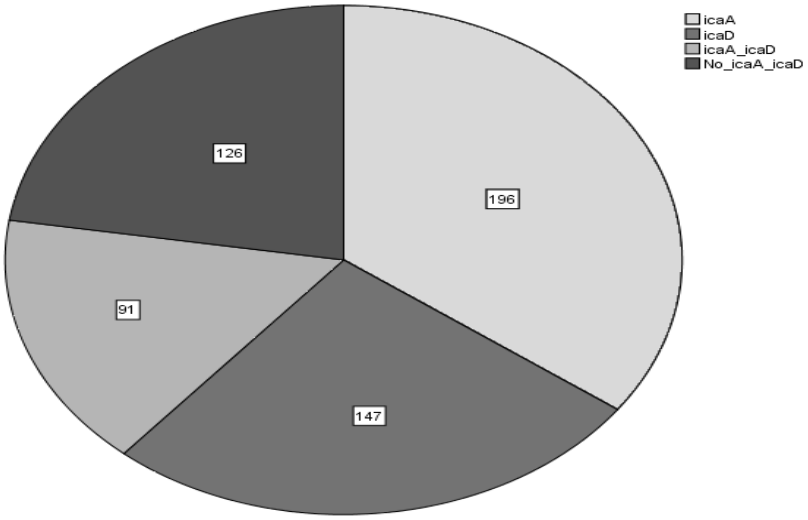

Figure 4.38. Frequency of *icaA* and *icaD* gene responsible for biofilm formation in all *S. aureus* isolates.

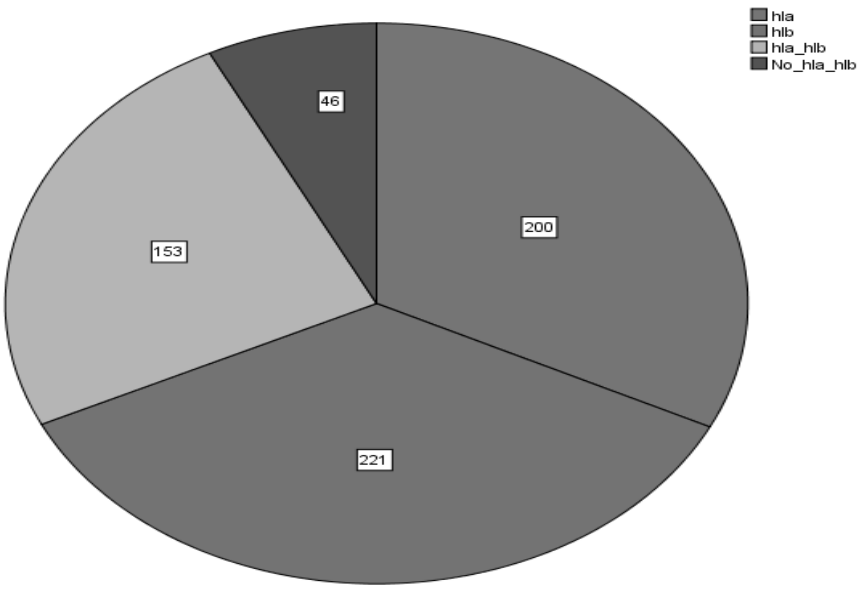

Figure 4.39. Frequency of *hla* and *hlb* gene producing phenotypial haemolysis in all isolates of *S. aureus*.

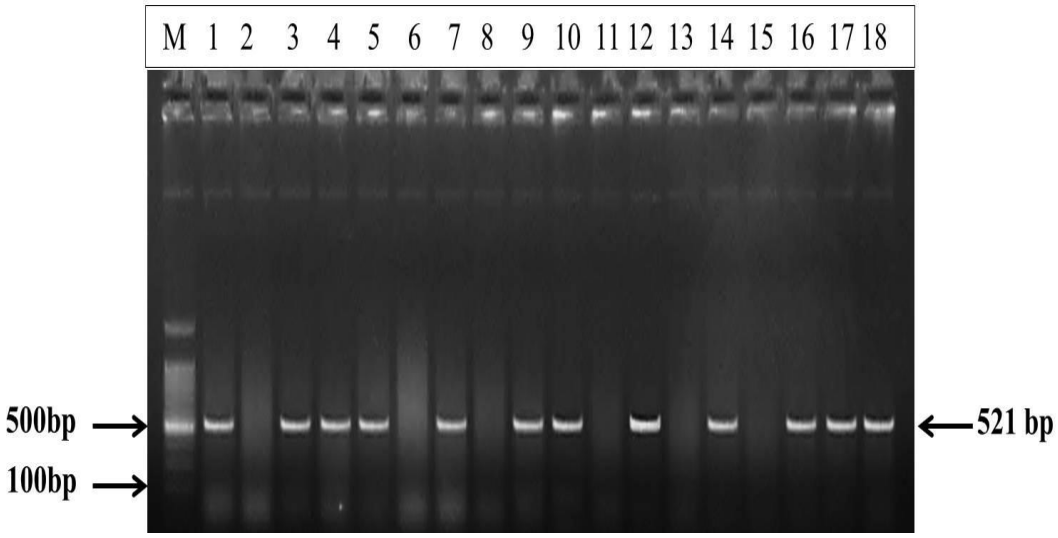

Figure 4.40. Molecular detection of *sea* gene. lane1= positive control, lane2= negative control, lane3-18= 521bp *sea* gene (isolate C15, C18, C41, C101, C39, CBM, C4075, C3074, C71P, ES22, E36, E52, E21, E109, E56, E91, EP52, ERM5 respectively M= 100bp marker.

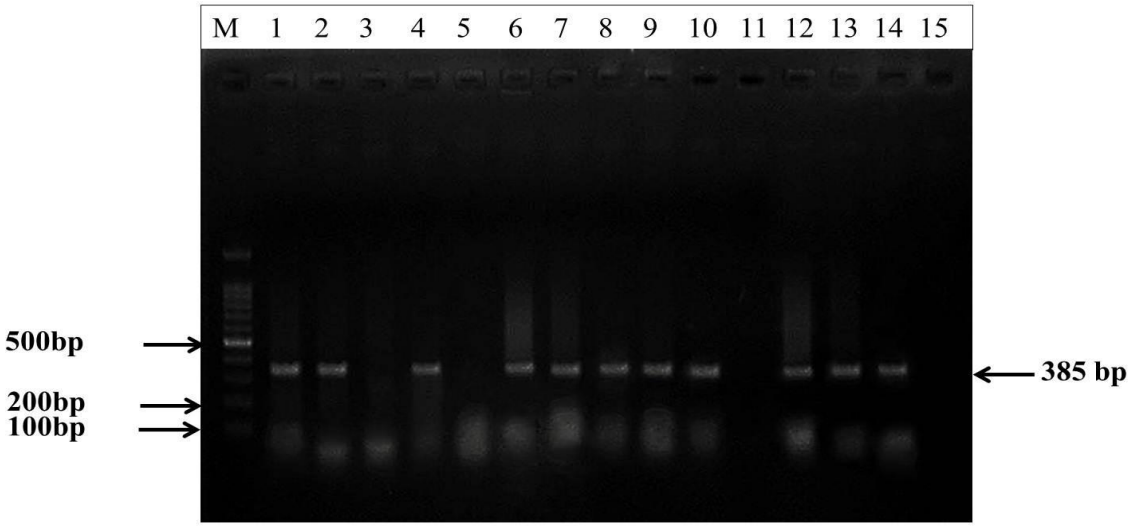

Figure 4.41. Molecular detection of *sed* gene. lane1= positive control, lane15= negative control, lane2-14= 385bp *sed* gene (isolate C19, CTS21, CP10, C1947, C29, C281, C199, EP22, EPM11, EN31, ES02, ENS4, E32, EK05 in lanes respectively) M= marker.

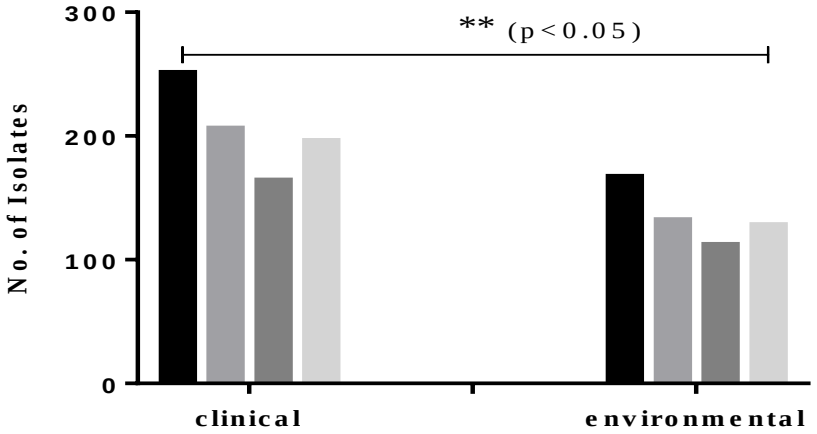

Figure 4.42. Frequency of extracellular enzyme production in clinical and environmental isolates of *S. aureus*. (p= 0.0019, t=10.32, df=3).
= thermonuclease, = protease, = lipase = lecithinase.

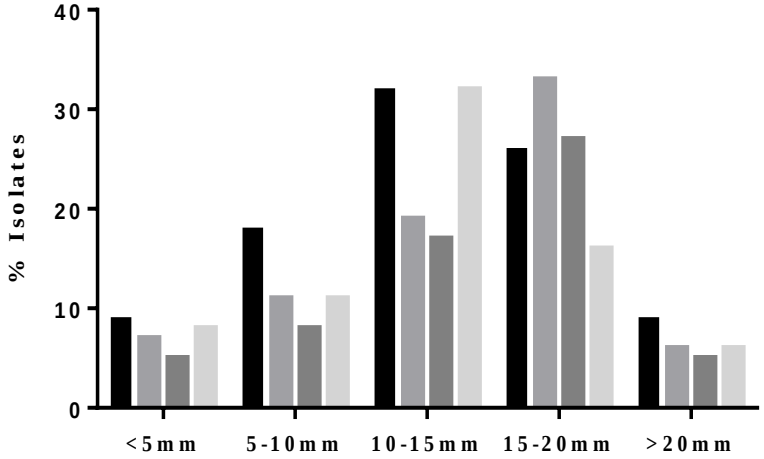

Figure 4.43. Graph showing zone diameter of extracellular enzyme production in all isolates of *S. aureus*.
= thermonuclease, = protease, = lipase = lecithinase.

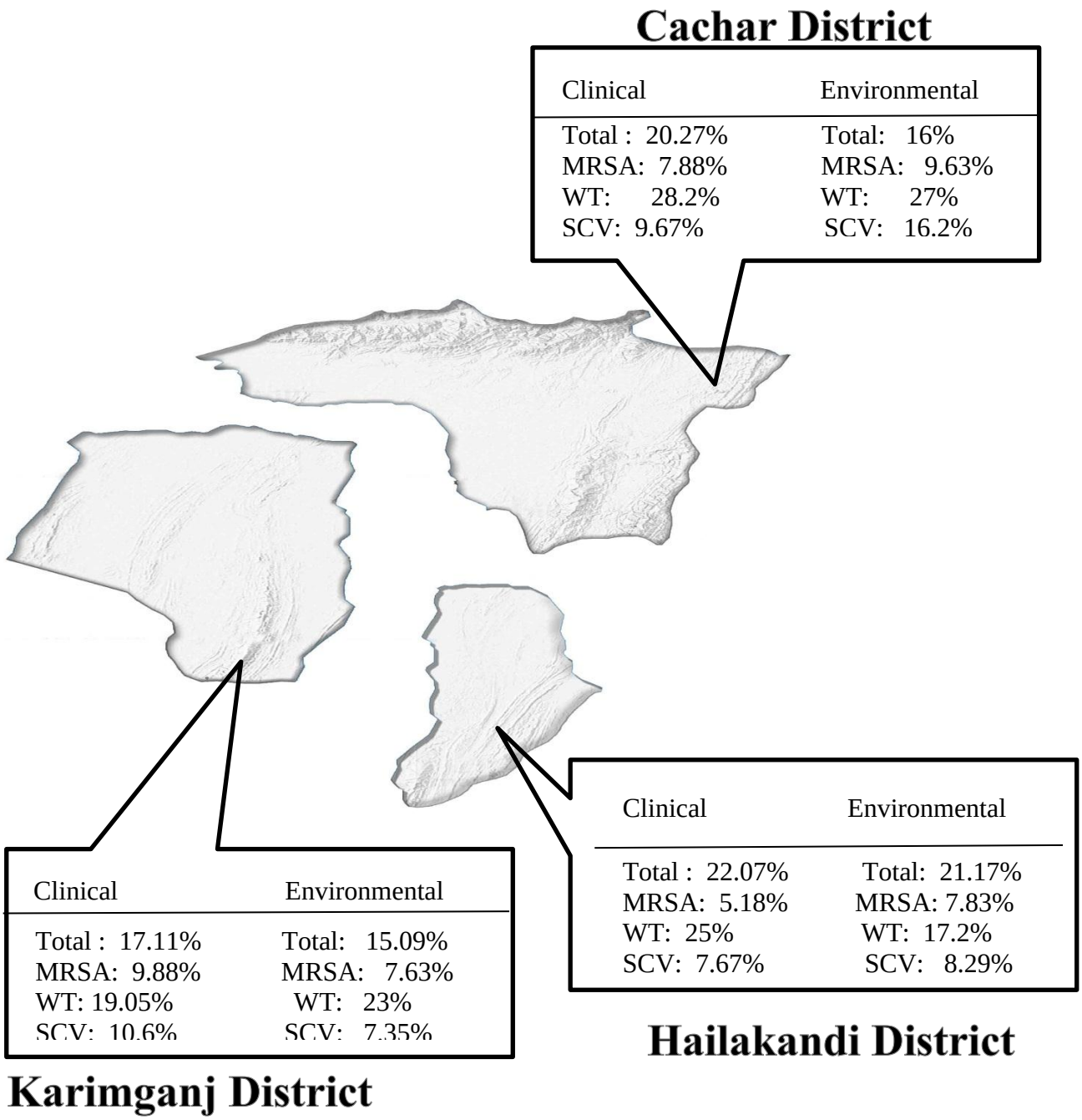

Figure 4.44. Comparison of region-wise prevalence of *S. aureus* isolates of south Assam alongwith their methicillin resistance profile.

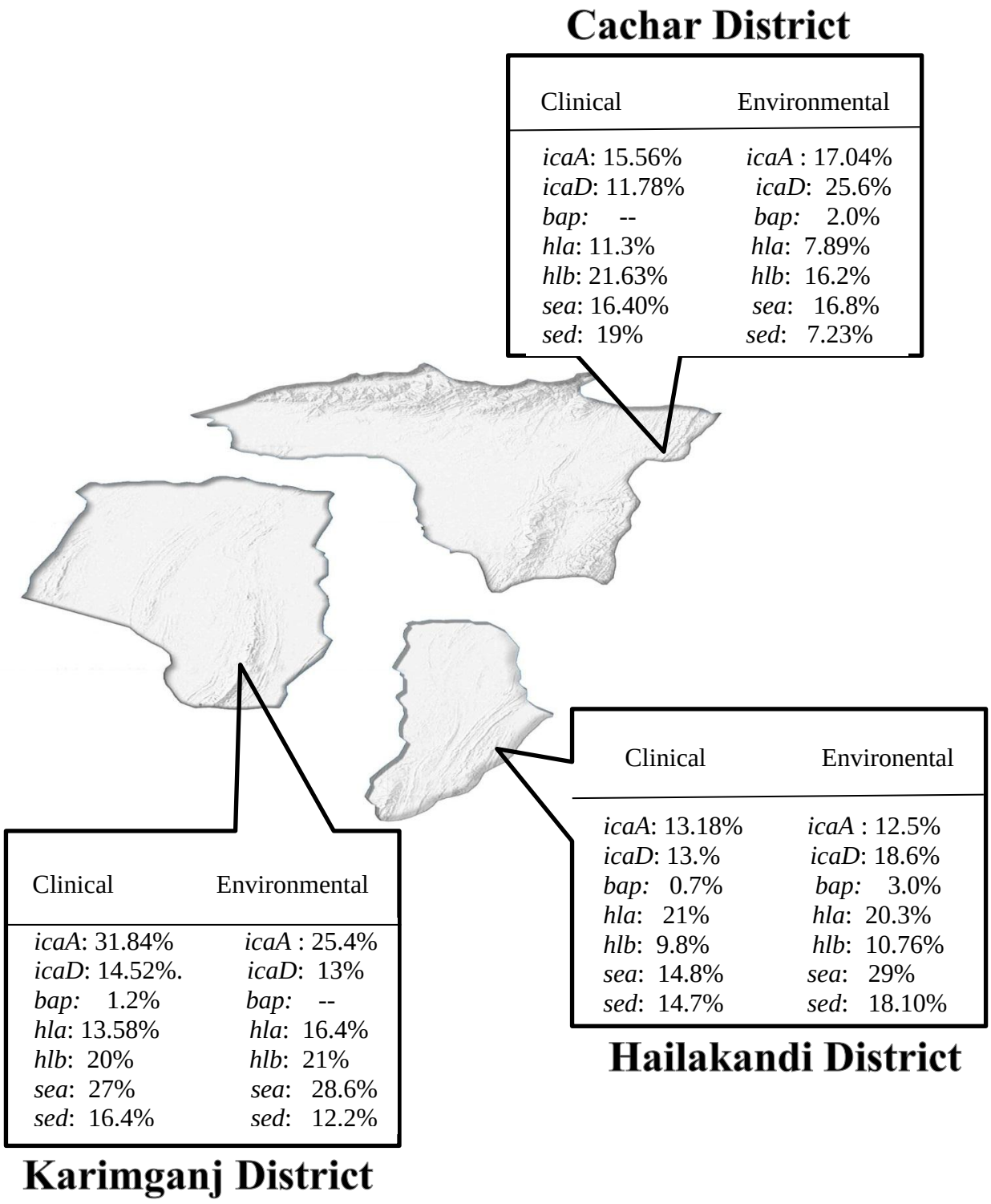

Figure 4.45. Region-wise prevalence of virulence factors of *S. aureus* isolates.