The biofilm-forming potential of *Staphylococcus aureus* isolates from various sources using phenotypic and genotypic assays

A Nirwan, M Shekhawat, D Suthar, Diwakar and AK Kataria

**Abstract**

*Staphylococcus aureus* is an important bacterial agent causing a variety of infections in humans and animals. This organism causes diseases like skin infections, meningitis, endocarditis, septicemia, and toxic shock syndrome. Biofilm production is considered one of the critical virulent factors that enhance survival of the organism and hence contributes to the pathogenic capabilities of this organism. In our study, a total of 40 *Staphylococcus aureus* isolates were obtained from different sources like pus and skin of humans and animals and unprocessed meat samples. The isolates were obtained by conventional microbiological methods, confirmed genotypically by 23S rRNA ribotyping and MALDI-TOF MS. The confirmed *S. aureus* isolates were screened for detection biofilm formation by Congo red agar method and for the detection of two genes responsible for biofilm production, namely *icaA* and *icaD* by a simplex polymerase chain reaction. Out of 42.50% and 32.50% isolates were detected as healthy and intermediate biofilm producer by the CRA method, and 100% and 97.56% isolates were exposed to possess *icaA* and *icaD* gene respectively by PCR. The very high prevalence of two genes in the isolates indicates the high pathogenic ability of these organisms as these genes are responsible for intracellular adhesions.

**Keywords:** *Staphylococcus aureus*, biofilm, *icaA* and *icaD* gene

**Introduction**

*Staphylococcus aureus* is gram-positive, spherical bacteria causing both community-associated and hospital-related infections [1]. It may cause various diseases like skin infections, meningitis, endocarditis, septicemia, and toxic shock syndrome. *S. aureus* has been studied widely for a better understanding of the molecular mechanisms that are involved in its pathogenicity. The invasive properties such as biofilm formation, adherence, and resistance to phagocytosis are characterized by the collective effect of extracellular factors and toxins of the pathogenesis of *S. aureus*. Biofilm producing bacteria are more adapted to survive the host immune response and antimicrobial therapy by decreased metabolism, reducing their growth and the penetration of antimicrobial compounds into the biofilm structure [3]. Bacterial biofilm is majorly composed of polysaccharides, teichoic acids, cell surface secreted bacterial proteinaceous adsions, extracellular DNA and other host plasma factors [4]. The biofilm formation mechanism is complex and requires the participation of many proteins, and so many genes are involved [5]. It is a two-step process: (i) attachment of bacterial cells to surface (ii) accumulation of bacteria in a multilayered set of intercellular adhesion [6]. Recent molecular studies revealed that during the last phase of adherence, bacteria first adhere to each other and then start elaborating the biofilm. This ability relies on the production of the extracellular matrix of *S. aureus*, composed of Poly-b (1,6) N-acetyl-D-glucosamine (Polysaccharide intercellular adhesion [PIA/PNAG]) which are produced by N-acetyl glucosaminyl transferase induced by the co-expression of the adhesion gene cluster i.e. *icaABCD* [7]. Among *icaABCD* cluster, the *icaA* and *icaD* genes have more important role in the biofilm formation rather than other genes. The *icaA* gene encodes N-acetyl glucosaminyl transferase and *icaD* has been identified to play a critical role in the high expression of N-acetyl glucosaminyl transferase, leading to the phenotypic expression of the capsular polysaccharide [6, 9, 10]. The present study was designed to detect the presence of biofilm-forming potential of two intracellular adhesives *icaA* and *icaD* genes in *S. aureus* isolates from various sources.
Material and Methods

Sample collection
A total of 82 samples were collected from various sources (Table 1) viz pus and skin of humans and animals and raw meat samples from different places in and around Bikaner (Rajasthan). Sterilized test tubes were used for sample collection and immediately transferred to the laboratory on ice for further processing.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Source</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Human pus</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Animal pus</td>
<td>15</td>
</tr>
<tr>
<td>3.</td>
<td>Skin of animal</td>
<td>14</td>
</tr>
<tr>
<td>4.</td>
<td>Skin of human</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Unprocessed meat</td>
<td>10</td>
</tr>
</tbody>
</table>

Isolation and identification of S. aureus
The isolated organisms were identified by MALDI TOF-MS and its cultural characteristics on mannitol soya agar and microscopic appearance in Gram-stained preparations and biochemical analysis were described [11, 12].

Biofilm formation assay
Congo red method was used for the detection of slime production from all S. aureus isolates as described [13]. Tryptic soya agar containing 0.08% Congo red (sigma) was used to prepared CRA Plates. The inoculated CRA plates were incubated at 37 °C in aerobic condition for 24 hours. The isolates were interpreted on the basis of their colony phenotype, as identified [14]. Black colonies of rough surfaces with dry consistency were considered a positive indication of slime production, while black colonies of round, glossy and smooth surface and red colonies of dry consistency and rough edges and surface were considered as intermediate slime producers.

Genotypic confirmation of S. aureus
Bacterial DNA isolation for PCR was done according to the method described [15] with some minor modifications. The genotypic confirmation of S. aureus based on 23S rRNA was carried out as per the described method [16]. The primer pairs used in PCR are depicted in Table 2. The reaction was carried out for 25μL of the final volume of PCR. The volume of isolated DNA used was 3μL. The primers were used at a concentration of 2pmol each. The Thermo scientific Dream Taq™ Green PCR Master Mix (2X) was used for PCR. The PCR cycle included initial denaturation at 95 °C for 1 min followed by 30 cycle of three steps (denaturation at 94 °C for 90s, annealing at 55 °C for 90s and extension at 72 °C for 75s) and final extension at 72 °C for 10 min.

Table 1:

<table>
<thead>
<tr>
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<th>Source</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>17</td>
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</tr>
<tr>
<td>3.</td>
<td>Skin of animal</td>
<td>14</td>
</tr>
<tr>
<td>4.</td>
<td>Skin of human</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Unprocessed meat</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2: Primers used for the detection of different genes in the current study.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>23S rRNA</td>
<td>F-5'-ACGGAGTATACAAAGGACGAC-3'</td>
<td>1250bp</td>
<td>Straub et al. (1999)</td>
</tr>
<tr>
<td>2.</td>
<td>icaA</td>
<td>F-5'-CCTAACTAAACGAGAAGGTAG-3'</td>
<td>1315bp</td>
<td>Vasudevan et al. (2003)</td>
</tr>
<tr>
<td>3.</td>
<td>icaD</td>
<td>F-5'-AAACGTAAGAGGGTG-3' R-5'-GGCAATTATGATCAAGATAC-3'</td>
<td>381bp</td>
<td>Vasudevan et al. (2003)</td>
</tr>
</tbody>
</table>

Multiplex PCR for detection of icaA and icaD genes
Multiplex PCR for the detection of icaA and icaD genes was done according to method as described [10]. The primer pairs used in PCR are depicted in Table 2. The reaction was carried out for 25μL of the final volume of PCR. The Thermo scientific Dream Taq™ Green PCR Master Mix (2X) was used for PCR. The primers were used at a concentration of 2pmol each. The PCR cycle included initial denaturation at 95 °C for 3min. 30 cycles of three steps (denaturation at 94 °C for 60s, annealing at 49°C for 60s and extension at 72°C for 60s) and a final extension at 72°C for 5min.

Table 3: List of samples which are positive for S. aureus

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of sources</th>
<th>Positive for S. aureus</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Human pus</td>
<td>7(17)</td>
<td>41.17%</td>
</tr>
<tr>
<td>2.</td>
<td>Animal pus</td>
<td>9(15)</td>
<td>60%</td>
</tr>
<tr>
<td>3.</td>
<td>Skin of animal</td>
<td>6(14)</td>
<td>42.85%</td>
</tr>
<tr>
<td>4.</td>
<td>Skin of human</td>
<td>10(26)</td>
<td>38.46%</td>
</tr>
<tr>
<td>5.</td>
<td>Unprocessed meat</td>
<td>8(10)</td>
<td>80%</td>
</tr>
</tbody>
</table>

Though S. aureus could be identified by conventional methods in the present investigation but the genotyping with a PCR based method involving specific primer targeted against 23S rRNA gene revealed an amplicon of 1250 bp (Fig. 1). All the 40 isolates confirmed to be S. aureus. This method was demonstrated by Straub et al. (1999) [16] and has been used by various researchers [17-24] for the identification of S. aureus from different sources.
CRA method was used for estimation of biofilm production for all 40 isolates. Out of these only 19(47.50%), 13(32.50%) and 8(20%) were strong, intermediate and weak or negative biofilm producers, respectively.

Prevalence of Adhesion genes icaA and icaD
In this study out of 40 S. aureus isolates 39 (97.56%) were found positive for icaA gene (Fig. 2) while all 40(100%) isolates have icaD gene was (Fig. 3). Khoramian et al. (2015)[25] made similar observations about the prevalence of icaA and icaD genes, who reported 88.4% icaD and 87.9% icaA among 215 isolates from human and dairy cow's infections. Similarly, 98% and 100% prevalence of icaA and icaD genes were also reported by Castelani et al. (2015)[26] in 110 isolates from heifers and cows with mastitis.
Similarly, Vasudevan et al. (2003) and Yazdani et al. (2006) [10, 27] were observed 100 % prevalence of both icaA and icaD genes from human clinical and bovine mastitis isolates. Ando et al. (2004) [28] reported that 99.1% isolates positive for icaD and while 77.1% of total human isolates were positive for clfA gene. Report of Xu et al. (2015) [29] were also similar to our findings. They found that the prevalence of clfA, clfB, icaD, agrI and agrII genes was 89.3%, 85.7%, 71.4, 64.3% and 17.9% respectively among 28 S. aureus isolated from cow mastitis milk but contrary to our finding they did not detect icaA, agrIII, and agrIV genes in any of the studied isolates. Likewise, high prevalence percentage was reported by many researchers viz; Atshan et al. (2012) [30] reported all 60 human isolates were positive for icaA and icaD. Bryan et al. (2013) [31] reported 100% presence of both the adhesion genes among six isolates of human. Similarly, Tang et al. (2013) [32] reported 87.50% presence of icaA and icaD genes among contaminated foods samples. Barbieri et al. (2015) [33] also observed the 100 % prevalence of icaA and icaD gene among 13 S. aureus isolates from breast peri-implant infections but Li-li et al. (2012) [34] reported slight lower prevalence in contrast to the present study, i.e. 31.3% presence of icaA and icaD gene among 137 isolates of bovine mastitis.

Conclusion
The present study revealed the presence of S. aureus strains carrying recently described adhesion genes from various sources. The studies of adhesion gene pattern and biofilm production in these S. aureus strains isolated from various sources will help in better understanding of the pathogenicity of this organism and consequently to control biofilm production. Furthermore, the presence of adhesion genes in S. aureus strains possess the potential public health concern and it indicates the necessity of monitoring these strains to discriminating specific population in order to identify the nature of the infection.

References
10. Vasudevan P, Nair MKM, Annamalai T,


