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A Nirwan
Ph.D Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance, Department of Microbiology and Biotechnology College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences, Dist. Bikaner, Rajasthan, India

M Shekhawat
M.V.Sc. Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Microbiology and Biotechnology College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences, Dist. Bikaner, Rajasthan, India

D Suthar
Junior Research fellow, Gene and Protein Engineering Lab, National Institute of Biotechnology Hyderabad, Telangana, India

Diwakar
Project Associate, Centre for Studies on Wildlife Management and Health, Department of Microbiology and Biotechnology College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Dist. Bikaner, Rajasthan, India

AK Kataria
Dean PGS, Rajasthan University of Veterinary and Animal Sciences, Professor & Head, Department of Microbiology and Biotechnology, O/I of Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan, University of Veterinary and Animal Sciences, Dist. Bikaner, Rajasthan, India

Corresponding Author:
A Nirwan
Ph.D Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance, Department of Microbiology and Biotechnology College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences, Dist. Bikaner, Rajasthan, India

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 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 which 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 produce 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* [2]. 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 proteinous adhesions, 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. *icaADBC* [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 [8, 9, 10]. The present study was designed to detect the presence of biofilm-forming potential of two intracellular adhesive *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 1: List of various sources used in the current study.

S. No.	Source	Total
1.	Human pus	17
2.	Animal pus	15
3.	Skin of animal	14
4.	Skin of human	26
5.	Unprocessed meat	10

Isolation and identification of *S. aureus*

The isolated organisms were identified by MALDI TOF-MS and its cultural characteristics on mannitol salt 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 ºC in aerobic condition for 24hours. 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 2: Primers used for the detection of different genes in the current study.

S. No.	Gene	Primer sequence	Size (bp)	Reference
1.	23S rRNA	F-5'-ACGGAGTTACAAAGGACGAC-3' R-5'-AGCTCAGCCTTAACGAGTAC-3'	1250bp	Straub *et al.* (1999)
2.	*icaA*	F-5'-CCTAACTAACGAAAGGTAG-3' R-5'-AAGATATAGCGATAAGTG C-3'	1315bp	Vasudevan *et al.* (2003)
3	*icaD*	F-5'-AAACGTAAGAGAGGTGG-3' R-5'-GGCAATATGATCAAGATAC-3'	381bp	Vasudevan *et al.* (2003)

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 DreamTaq™ 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.

Agarose gel electrophoresis

The amplify PCR product of 23S rRNA, *icaA* and *icaD* were analyzed by electrophoresis on 0.8% and 1% agarose gel, respectively.

Results and Discussion

Detection of phenotypic and genotypic analysis

In this study, out of 82 samples, only 40 samples were found positive for *S. aureus*. The colonies of 40 *S. aureus* isolates showed golden yellow pigmentation on nutrient agar and fermented mannitol on mannitol salt agar. Further confirmation of 40 *S. aureus* isolates was done by MALDI-TOF MS.

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

S. No.	Name of sources	Positive for *S. aureus*	%
1.	Human pus	7(17)	41.17%
2.	Animal pus	9(15)	60%
3.	Skin of animal	6(14)	42.85%
4.	Skin of human	10(26)	38.46%
5.	Unprocessed meat	8(10)	80%

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.

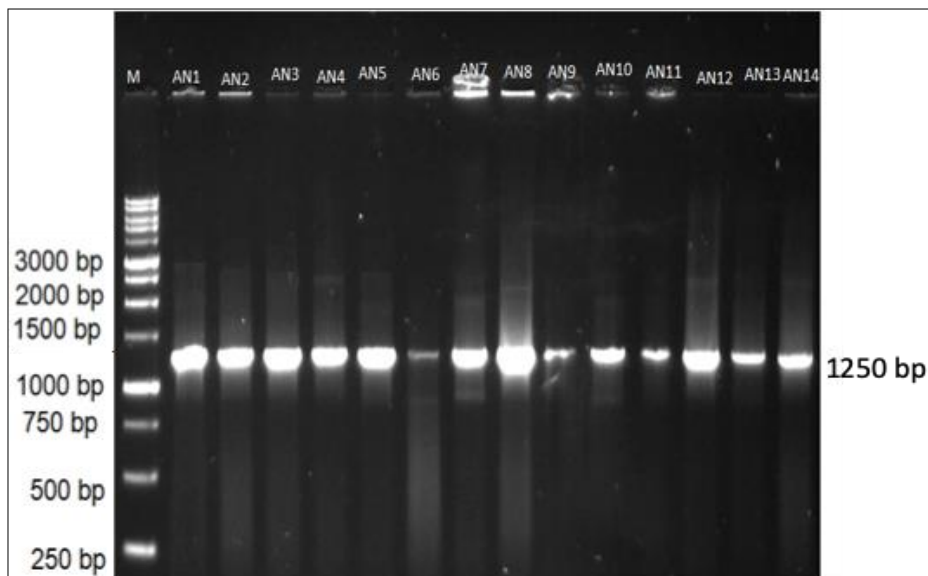

Fig 1: Agarose gel electrophoresis of PCR product of 23S rRNA ribotyping of *S. aureus* isolates; M – Molecular marker (1250bp); AN 1-AN14: Isolates from various sources.

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 i*caD* genes were also reported by Castelani *et al*. (2015) [26] in 110 isolates from heifers and cows with mastitis.

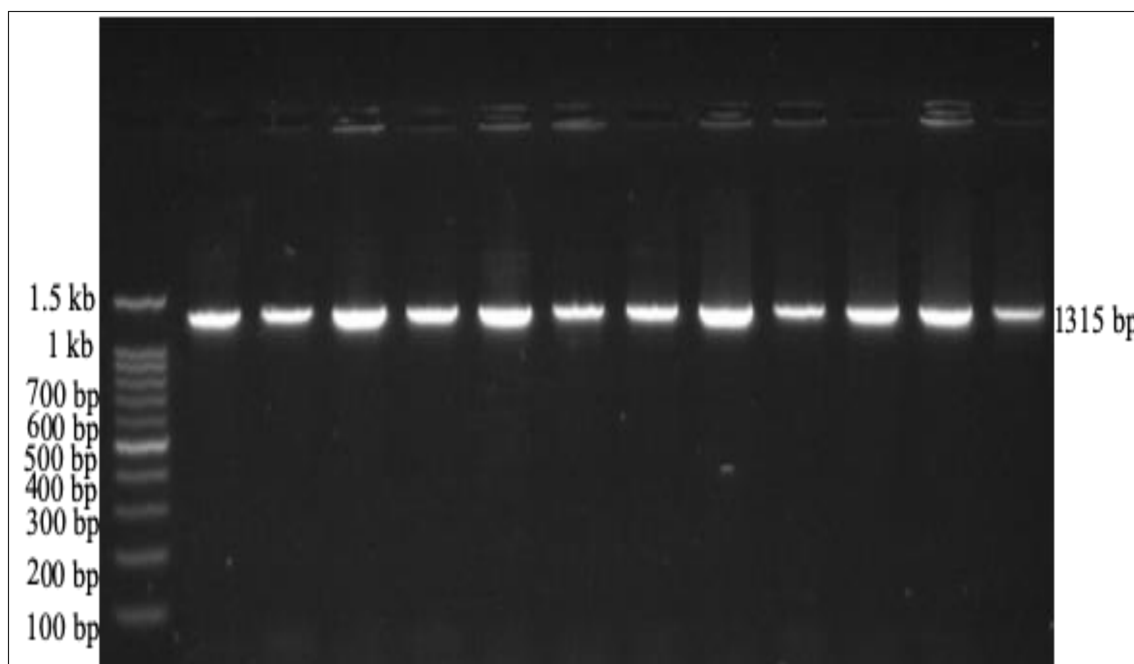

Fig 2: Agarose gel electrophoresis of *icaA* gene amplicon of *S. aureus;* M – Molecular marker (1315bp); AN1- AN12: Isolates from various sources.

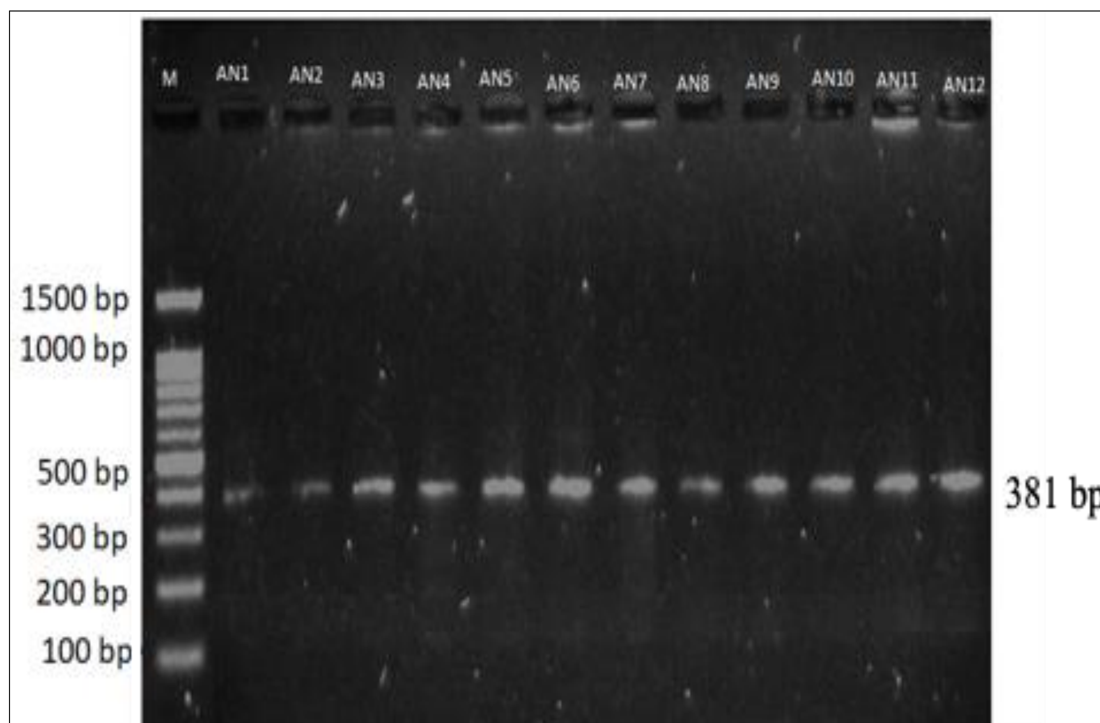

Fig 3: Agarose gel electrophoresis of *icaD* gene amplicon of *S. aureus;* M – Molecular marker (381bp); AN1- AN12: Isolates from various sources.

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.* Bnyan *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.

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