ORIGINAL ARTICLE

Identification of Virulence Factors, Accessory Gene Regulator (agr) Locus and Antibiotic Resistance Pattern of Staphylococcus aureus Isolated from Diabetic Foot Ulcers

1Rania Talaat Abdel Haleem, Msc.*, 1Magda Mohammad EL Nagdy, MD., 1Nesreen Salah Omar, MD., 2Mamdouh Radwan El-Nahas, MD. 1Rawia Ibrahim Badr, MD.
1Department of Medical Microbiology and Immunology; 2Department of Internal Medicine, Faculty of Medicine, Mansoura University, Egypt

ABSTRACT

**Background:** Staphylococcus aureus (S. aureus) is a common virulent pathogen in diabetic foot infections. Infection of diabetic foot ulcer (DFU) is a major cause for impaired healing and leads to osteomyelitis, bacteraemia and sepsis. Treatment failure of DFU may ultimately lead to limb amputation. **Objectives:** This work was carried out to clarify the role of S. aureus as a causative agent of diabetic foot infection, and characterize the antibiotic resistance pattern, virulence factors implicated in the pathogenicity of S. aureus and the accessory gene regulator as a global regulator of virulence determinant production. **Methodology:** This study was carried out on 111 patients admitted to diabetic foot clinic (MUH) having infected diabetic foot ulcer. Grading of infection was assessed. The collected specimens were cultivated on 5% sheep blood agar and mannitol salt agar. Isolates were identified as S. aureus by being catalase positive, mannitol fermenter, DNase positive and coagulase positive. Virulence was characterized by thermonuclease test, slime production, hemagglutination test, and biofilm formation. Multiplex PCR was used for detection of agr groups. **Results:** S. aureus represented 32% of total pathogens isolated. 58.3% of S. aureus strains were slime producers. 70.8% were biofilm producers. The most prevalent agr type was agr 1 and represented 54.2% followed by agr 2 (29.2%), agr 3 (8.3%), and agr 4 (4.2%). **Conclusion:** S. aureus is the most common cause of diabetic foot infection at our locality. Slime and biofilm producing strains are more resistant to antibiotics than non producers. S. aureus infection is more common among grade 2 and 3 ulcers. This data is crucial for selection of appropriate antibiotic therapy. agr type 1 is the most prevalent type in DFU at our locality.

INTRODUCTION

*S. aureus* is by far the most common and most virulent pathogen in diabetic foot infections. Most recent studies on the microbiology of diabetic foot infection have originated from Asian and African countries. The majority of these have shown that *S. aureus* remains the single most commonly isolated species. Because of the dramatically increased risk of amputation due to foot infection in diabetic individuals, early diagnosis and adequate treatment are essential.

---

*Corresponding Author:*  
Rania Talaat Abdel Haleem  
Department of Medical Microbiology and Immunology  
Faculty of Medicine, Mansoura University, Egypt  
E-mail: rantalaat@yahoo.com

The pathogenicity of *S. aureus* infections is related to various bacterial surface components (e.g., capsular polysaccharide and protein A). These include those recognizing adhesive matrix molecules (e.g., clumping factor and fibronectin binding protein), and adhesives extracellular proteins (e.g., coagulase, hemolysins, enterotoxins, toxic-shock syndrome [TSS] toxin, exfoliatins, and Panton-Valentine leukocidin [PVL]). *agr* locus controls virulence factors. The locus is a quorum-sensing gene cluster of five genes (*hld, agrB, agrD, agrC, and agrA*). It up-regulates production of secreted virulence factors, including the alpha-, beta- and delta-hemolysins, and down-regulates production of cell-associated virulence factors.

Polymorphisms in *agrD* and *agrC* define four *S. aureus* agr groups (designated agr-lSa to agr-4Sa). Within each group, all strains produce a peptide that can
activate the agr response in the other members of the same group. Auto-inducing peptides are usually mutually inhibitory between members of different groups. Different agr groups, as defined by their production and recognition of distinct secreted signals, are associated predominantly with certain diseases. Although most human clinical S. aureus isolates are agr+, there have been several reports of agr-defective mutants isolated from infected patients. The association between agr-specific groups and infection type has been reported by Jarrad and colleagues.

Wound-associated biofilms result in chronic and often non-healing infections, some leading to death. A study by Yarwood et al.,9 raised the possibility that the agr quorum-sensing system is involved in biofilm detachment. Boles and Horswill,10 indicated that induction of the agr system; in established S. aureus biofilms; detaches cells. The dispersal mechanism requires extracellular protease activity.

Other important correlation between agr and antibiotic resistance pattern, was observed. Compromised agr function is advantageous to clinical isolates of S. aureus toward the development of vancomycin hetero- resistance. Also agr grouping among S. aureus isolates with reduced susceptibility to glycopeptides belong to accessory gene regulator group I or II. The Infectious Diseases Society of America/International Working Group on the Diabetic Foot (IDSA) classification scheme for diabetic foot ulcers includes four progressive levels of infection based upon severity correlated to clinical findings.

Sotto et al.,3 found that the virulence genes of S. aureus were present significantly more in wound with grades 2–4 ulcers than grade 1. In addition, the presence of virulence factors whether at presentation or at follow-up in patients with clinically uninfected wounds is predictive of a poor clinical outcome. Thus, determining virulence genes may help to differentiate non infected (grade 1) from infected (grade 2–4) wounds.

This study was carried out to isolate S. aureus from infected DFU in our locality, investigate agr type among different grades of ulcer infection and characterize virulence factors and antibiotic resistance pattern of the isolated S. aureus.

METHODOLOGY

Study design: This study was conducted over a period of 20 months from July 2012 to February 2014 on patients with infected DFUs attending diabetic foot clinic of Mansoura University Hospital (MUH).

Study population: This study was carried out on 111 patients with infected DFUs.

Data collection: The following data were collected: demographic characteristics of the patients (name, age, sex), type and duration of diabetes, treatment for diabetes, hypertension and antimicrobial therapy.

Foot examination: to diagnose peripheral neuropathy and vasculopathy.

Ulcer assessment: included; size, site, duration, clinical signs of infection and grade of the ulcer infection. Classification and severity of diabetic foot infections was done according to the Infectious Diseases Society of America/International Working Group on the Diabetic Foot, grade 2-4 ulcers were included that represented mild, moderate and severe infection.

Clinical samples: A total of 111 clinical samples were collected after wound debridement. Samples for bacterial culture were obtained by tissue biopsies.

Microbiologic studies: Samples were processed at Microbiology Diagnostics and Infection Control Unit (MDICU) in Medical Microbiology and Immunology department, Faculty of Medicine, Mansoura University. The collected samples were cultivated on 5% sheep blood agar, chocolate agar and Mac Conkey agar incubated aerobically at 37 °C for 48 h. The colonies with morphologies compatible with Staphylococcus spp., were sub-cultured on mannitol salt agar (Oxoid). S. aureus was identified by Gram stain, colony morphology, biochemical reactions by being catalase positive, mannitol fermenting, DNase positive and were confirmed by coagulase test (bioMérieux).

Antibiotic Susceptibility Testing: Antimicrobial susceptibility of the identified S. aureus isolates was performed using disk diffusion method on Mueller–Hinton agar to determine its sensitivity to different antibiotics.

Virulence characterization: Includes Thrommonuclease (TNase) test for the detection of TNase activity, congo red agar for detection of slime production, hemagglutination test performed in Ushaped 96-well microtiter plates, and detection of biofilm formation by S.aureus strains on polystyrene was quantified using the microtiter plate assay.

Multiplex PCR for detection of agr groups:

A- Chromosomal DNA extraction: Genomic DNA extraction was carried out for S.aureus isolates using QIAamp® DNA Mini kits, QIAGEN (Germany). Additional reagents required for Gram-positive was 20 mg/ml lysozyme solution (Sigma) in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1.2% Triton.

B- agr group-specific multiplex PCR: The following primers were used: Pan (5′-ATG CAC ATG GTG CAC ATG C-3′), agr 1 (5′-GTC ACA AGT ACT ATA AGC TGC GAT-3′), agr 2 (5′-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3′), agr 3 (5′-GTA ATG TAA TAG CTT GTA TAA TAC CCA G-3′), and agr 4 (5′-CGA TAA TGC CGT AAT ACC CG-3′). These primers allow the amplification of a 441-bp DNA fragment of the agr group 1 strains, of a 575-bp DNA fragment of the agr group 2 strains, of a 323-bp DNA fragment of the agr group 3 strains, and of a 659-bp
DNA fragment of the agr group 4 strains. Amplification was done through the following temperature program: predenaturation: 1 cycle at 94°C for 5 minutes, then 26 cycles were performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds and post extension by incubation at 72°C for 10 min. 21

C- Detection of the agr types gene bands:
Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide on comparison to DNA standard marker: 50 bp DNA Ladder #SMO373 (Thermo Scientific Inc.) and visualized by transillumination under UV. 21

Statistical Analysis:
Computer SPSS program version 18 was used in Windows 8.0. Categorical data were analyzed using chi-square test to study the significance between 2 groups. The results were expressed by applying Chi-square test and P value. All these tests were considered significant if p value < 0.05.

RESULTS
The study included 111 patients, after exclusion of 2 cases recorded as no growth, the total number of cases was 109. 149 strains were isolated from 109 samples, corresponding to a mean number of 1.37 isolates per sample. A polymicrobial infection was present in 40 samples. In 69 samples infection was monomicrobial. S. aureus was the most common organism isolated. It represented 32% of all the isolates.

From the isolated S. aureus strains, 41.7% were grade 2 ulcer, 50% were grade 3, and 8.3% were grade 4. Among the other isolates the grade 2 ulcer was present in 37.7%, grade 3 in 26.2%, grade 4 in 36.1%. These results were statistically significant with P value 0.002.

The risk factors that contribute to diabetic foot infection, include hypertension, neuropathy and vasculopathy. 57.8% of patients with isolated S. aureus had hypertension, 79.8% had peripheral neuropathy, and patients with peripheral vascular disease represented 44%. All isolates of S. aureus were positive for coagulase, DNase test, thermonuclease test.

Antimicrobial susceptibility testing of S. aureus isolates revealed that S. aureus strains were most sensitive to vancomycin with sensitivity 87.5% and to piperacillin/tazobactam with sensitivity 83.3%. S. aureus strains were most resistant to penicillin and ampicillin with resistance 91.7%.

Resistance to augmentin was 58.3%, gentamycin 62.5%, cloramphenicol 45.8%, ofloxacin 66.7%, cefuroxime 58.3%, tobramycin 54.2%, erythromycin 45.8%, trimethoprim/sulphamethoxazole 66.7%, and imipenem 45.8%.

Out of 48 S. aureus isolates 15 were MRSA and represents 31% of S. aureus isolates and 10% of all isolates. Six isolates were vancomycin resistant (VRSA) and represents 12.5% of isolated S. aureus and 4% of all isolates.

Thermonuclease test was positive in all isolates and hemaglutination test was positive in 25% of isolates.

Slime production on congo red agar revealed that 58.3% of S. aureus strains were slime producers, and 41.7% were non slime producers. Slime positive strains are more resistant to antibiotics than slime negative strains. The results were statistically significant in case of augmentin with P value 0.001, tetracycline with P value 0.005, and methicillin with P value = 0.000.

Biofilm producing strains were more prevalent among the isolated S. aureus strains than non-biofilm producers with prevalence rate 70.8%. 29.2% of isolated S. aureus strains were non biofilm producers, 35.4% were moderate biofilm producers, and 35.4% were strong biofilm producers. Biofilm positive strains were more resistant than biofilm negative strains. The difference was statistically significant for amoxicillin/potassium clavulanate (P value = 0.009), tetracycline (P value = 0.000), and methicillin (P value = 0.002).

As regard the prevalence of agr specificity groups among S. aureus isolates, our study revealed that 54.2% of isolated S. aureus were agr type 1, 29.2% were agr type 2, 8.3% were agr type 3, 4.2% were agr type 4, and 4.2% were non type-able.

Table 1: Correlation between agr type and the grade of ulcer infection

<table>
<thead>
<tr>
<th>Grade of ulcer infection</th>
<th>agr typing</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>agr1</td>
<td>agr2</td>
</tr>
<tr>
<td>grade 2</td>
<td>number</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>60</td>
</tr>
<tr>
<td>grade 3</td>
<td>number</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>50</td>
</tr>
<tr>
<td>grade 4</td>
<td>number</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>number</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>54.2</td>
</tr>
</tbody>
</table>
The table shows that agr type 1 represented 60% of grade 2 ulcers, 50% of grade 3 ulcers and 50% of grade 4 ulcers, thus it is the commonest type isolated from grades 2 and 3 ulcers. agr type 2 strains represented 20% of grade 2 ulcers, 33.3% of grade 3 ulcers and 8.3% of grade 4 ulcers. agr type 3 represented 10% of grade 2 ulcer, 8.3% of grade 3 ulcers and not detected in grade 4 ulcers. agr type 4 was detected only among grade 3 ulcers and represented 8.3%. The non type-able strains represented 10% of all isolates.

The present study showed that the prevalence of \textit{S. aureus} isolated from diabetic foot patients is 32%. Others have reported a prevalence as low as 19.5% \cite{26} and as high as 32%. \cite{27}

In this study, 57.8% of the patients had hypertension. An incidence that is higher than 45.3% as documented by Abdulrazak et al. \cite{28} but much lower than 81.5% as documented by Candel Gonzalez et al. \cite{29}

In our study 79.8% of patients had peripheral neuropathy. Sotto et al., \cite{14} reported a nearly similar incidence of 81%. Conversely, Candel Gonzalez et al. \cite{29} reported a much lower incidence of 21%. In this study, patients with peripheral vascular disease represented 44%. Candel Gonzalez et al. \cite{29} and Sotto et al. reported similar incidence of 27% and 39% respectively. \cite{14}

Out of 48 \textit{S. aureus} isolates; 15 were \textit{methicillin resistant S. aureus (MRSA)} and represent 31% of \textit{S. aureus} isolates and 10% of all isolates. Six isolates were vancomycin resistant (\textit{VRSA}) and represent 12.5% of isolated \textit{S. aureus} and 4% of all isolates. Others have reported slightly higher \textit{MRSA} rates of 41% and 46%. \cite{14,30}

In our study, 41.7% of \textit{S. aureus} isolates were grade 2 ulcers, 50% grade 3, and 8.3% grade 4. Djahmi et al. \cite{31}, documented that 7% of \textit{S. aureus} isolates were grade 2 ulcers, 64.7% were grade 3, and 10.6% were among grade 4 ulcers. Thus, they have a higher rate of higher grade ulcers.

In our study, most mild to moderate infections are caused by \textit{S.aureus}. Deeper limb-threatening infections were usually caused by aerobic gram-positive cocci and gram-negative bacilli. The high percentage of \textit{Staphylococcus} species in the present study may be attributed to the high rate of superficial diabetic foot infections (DFIs). The same finding was documented by El-Tahawy et al. where the majority of DFIs were superficial and were frequently colonized by aerobic gram-positive bacteria. \cite{35}

Hemagglutination test was positive in 25% of \textit{S. aureus} isolates in our study. However, Rupp et al. \cite{32} reported much lower incidence of only 13%.

In this study, antimicrobial susceptibility testing revealed that, \textit{Staphylococcus aureus} isolates showed highest sensitivity to vancomycin and piperacillin/tazobactam with sensitivity 87.5% and 83.3% respectively and lowest sensitivity to penicillin and ampicillin with sensitivity 8.3%.

Vancomycin resistance was 12.5% in our study. Others have reported a much high resistance of 63% and 70%.\cite{33,34} Conversely others have reported a much lower resistance of 0%\cite{28}. Penicillin and ampicillin resistance was 91.7% in our study. The same rates of such high resistance was documented by others. El Tahawy et al. \cite{26} and Shanmugam et al. \cite{35} reported similar high resistance rates of 90% and 100% respectively. The high resistance to penicillins is
attributed to the indiscriminate use of this older group. Cefuroxime resistance was 48% which is nearly similar to 50% incidence as reported by Swarna et al. 27 Ofloxacin resistance was 66%, which is nearly similar to 69% incidence as reported by Swarna et al. 27 45.8% of S. aureus were resistant to erythromycin in our study. Others reported variable incidence of erythromycin resistance of 16 and 70%. 26,36

Gentamycin resistance was 62.5% in our study. El Tahawy et al. 26 reported a higher incidence of 77%. Whereas, Djahmi et al. documented a resistance rate nearly similar to ours of 59.4%. 31 66.7% of isolated S. aureus strains were resistant to trimethoprim/sulphamethoxazole. A very similar value of 60% was reported by Hena and Growther. 34 Swarna et al. 27 reported a much lower value of 37.5%.

Augmentin resistance was 58.3% in our study. A value that is higher than reported by Abdulrazak et al. 28 Tetracycline resistance was 54.2% that is much lower than that of 89.4% as reported by Hena and Growther 34 Of our S. aureus isolates 45.8% were resistant to chloramphenicol and higher than 30% as reported by Hena and Growther. 34

Slime production is a very important factor related to biofilm formation. 58.3% of S. aureus strains were slime producer, Podbielska et al. 37 studied slime production in S. aureus isolated from diabetic foot ulcers, they reported that 69% of S. aureus strains produced slime as shown by the CRA method.

Among S. aureus isolates 70.8% were biofilm producers and 29.2% were non biofilm producers. Swarna et al., 27 reported that among S. aureus isolated from diabetic foot ulcers 50% were biofilm positive.

Our study demonstrated that biofilm producers are more resistant to antibiotics more than non biofilm producers.

As regard the prevalence of agr specificity groups among S. aureus isolates, our study revealed that 54.2% of isolated S. aureus were agr type 1, 29.2% were agr type 2, 8.3% were agr type 3,4.2% were agr type 4, and 4.2% were non typeable. Traber et al. 8 revealed that 50% of their study group had an agr 1 strain, 34 (33.3%) agr 2 , 14 (13.7%) agr 3, and 1 (0.98%) agr 4. They reported that the distribution of agr types among the isolates doesn't represent a significant departure from the overall results from other series, and they didn't encounter any correlation between clinical features and agr type.

S. aureus agr typing by other investigators has generated widely varying distribution of agr types. It was reported that agr distribution differ according to different geographical areas, many studies were congruent with our study as regard the predominance of agr 1 type. In USA, a study done by Shopsin et al., reported that 42% of their collected samples belonged to agr group I, followed by group III (34%) and group II (24%), they reported that these findings seem to reflect the natural distribution of lineages in this geographical area according to agr groups and are similar to findings of other studies. 38

Some investigators reported that there is correlation between agr groups and infection type. 6 On the other hand, Yarwood and Schlievert reported that the reasons for the association between agr groups and infection types are not yet clear, but a better understanding of this phenomenon may contribute to the understanding of the epidemiology of staphylococcal diseases. 39

Traber et al. 8 reported that agr-negative variants arise commonly in vivo as well as in vitro, the occurrence of such organisms in clinical material does not mean that they actually initiated the infection. In fact, agr-negative strains have not been shown to initiate infections, and it is suggested that they may be genotypic dead-ends in the ecology of the organism.

Our results demonstrated that 60% of grade 2 ulcers were classified as agr 1 type, 20% as agr 2, 10% as agr 3, and 10% were non typeable. 50% of grade 3 ulcers were among agr 1, 33.3% were agr 2, 8.3% were agr 3 50% of grade 4 ulcers were among agr 1, and 50% were agr 2. No statistically significant difference between the different types of ulcers.

Our results go with the results of Sotto et al. 14 who reported that 69% of grade 2 ulcers were classified as agr type 1, 14% as agr type 2, 11% as agr type 3, and 6% were agr type 4. 47% of grade 3 ulcers were among agr type 1, 24% were agr type 2, 16% were agr type 3 and 13% were agr type 4. 50% of grade 4 ulcers were among agr type 1, and 50% were agr type 2. They mentioned that these results were not statistically significant.

CONCLUSION

S. aureus is the most prevalent pathogen causing diabetic foot infection in our locality and represents 32% of isolated pathogens. S. aureus infection was more common among grade 2 and grade 3 ulcers that represents mild to moderate infection. Slime production among S. aureus isolates represented 58.3% and biofilm production represented 70.8%. Antibiotic resistance was more common among slime producing and biofilm producing isolates than non producers. agr typing of S. aureus isolates revealed that agr 1 is the most prevalent type in our locality. There was no significant correlation between the grade of ulcer infection and the agr type.

REFERENCES


