Hovedoppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for medisin og helsevitenskap

Nora Moen Vadet og Åsta Hereid Ringheim

Elements in *Staphylococcus aureus* associated with endocarditis

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Veileder: Ingvild Haugan
Medveileder: Jan Egil Afset og Christina Gabrielsen Ås
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Students

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Abstract

Background: Infective endocarditis (IE) is a dreaded infectious disease due to its heterogeneous course, the challenges of reaching a diagnosis and its high mortality. *Staphylococcus aureus* is the most frequent causative organism in high income countries. Some studies suggest that characteristics of the infecting strain can predict the severity of IE.

Objectives: The aim of this study was to compare the presence of a selection of genes (*clfA*, *clfB*, *fnbA*, *fnbB*, *sspA*, *sspB*, *cna*, *sdrC*, *sdrD*, *sdrE*, *isdA* and *vWbp*) and adherence to fibrinogen or fibronectin of *S. aureus* strains from asymptomatic carriers and patients with IE. We also investigated the association between the bacterial genotype, the ability to adhere in an adherence assay (*in vitro* phenotype) and the ability to cause endocarditis (*in vivo* phenotype).

Methods: The adherence of 27 *S. aureus* carriage strains and 30 *S. aureus* IE strains to solid-phase fibrinogen and fibronectin was assessed using a microtiter assay, and compared to the adherence of a control strain (*S. aureus* 8325-4) used as reference. Data about the strains from a previously performed whole-genome sequencing (WGS) were used to evaluate sequence types (ST), clonal complexes (CC) and the presence of 12 genes known to encode proteins involved in adherence.

Results: The fibrinogen and fibronectin adherence values are given as the percentage of the mean optical density (OD) value of *S. aureus* 8325-4 after subtracting the mean OD value of the negative controls. Both carriage strains and IE strains showed variability in adherence values. The fibrinogen adherence values ranged from 16% to 162% for the carriage strains, and from 21% to 220% for the IE strains. The fibronectin adherence values ranged from -4% to 312% for the carriage strains, and from -11% to 284% for the IE strains. The adherence values between carriage and IE strains did not differ significantly for neither fibrinogen (p=0.117) nor fibronectin (p=0.114). Four genes that encode proteins involved in adherence (*clfA*, *fnbA*, *fnbB*, *sdrC*) were significantly more common among the IE than the carriage strains.

Conclusions: We found an association between being an IE strain and three genes that code for adhesins to fibrinogen, fibronectin, or both (*clfA, fnbA* and *fnbB*) and one gene (*sdrC*) involved in adhesion through unknown mechanisms. The mean adherence values to both fibrinogen and fibronectin in the *in vitro* adhesion assay were similar between IE and carriage strains. This may suggest that this *in vitro* assay is not suitable for assessment of *S. aureus in vivo* adhesion or that the adhesion to fibrinogen or fibronectin is not a distinguishing feature of IE strains. In future studies other methods to assess for adherence in *S. aureus* should be investigated, and we recommend further research on other bacterial characteristics that may be important for the development of IE.

Introduction

Infective endocarditis

Infective endocarditis (IE) is an infection of the endocardial surface of the heart. The nidus is often on a native or prosthetic heart valve or an indwelling cardiac device (1). The incidence of the disease is estimated to be between 1.5 to 6 per 100.000 person-years based on previous studies in Europe and the United States (2). Accurate incidence numbers are difficult to obtain due to the challenge of reaching a diagnosis, as IE is heterogeneous in aetiology, clinical manifestations, and disease course (3). Diagnostic criteria, such as the Duke criteria for infective endocarditis, help assess patients with suspected IE. Some diagnostic criteria have a lower sensitivity for patients with prosthetic valve endocarditis or cardiac device infection (3). Infective endocarditis is associated with a high mortality (4), and S*taphylococcus aureus* is the most frequent causative organism in high income countries where it accounts for 16-34% of all IE cases (2).

Staphylococcus aureus

S. aureus is a commensal bacterium found in the nose, upper respiratory tract or skin in around 20% of the population (5). However, the bacterium is also a human pathogen that can cause a broad spectrum of clinical infections. Other than being a leading cause of IE, *S. aureus* is often found in bloodstream infections (BSI) and a frequent cause of skin, soft tissue, osteoarticular and device-related infections (2). *S. aureus* harbours a vast variety of virulence factors and can acquire resistance to antibiotics making it difficult to treat infection caused by this bacterium (6).

The role of *S. aureus* virulence associated with endocarditis

Several *S. aureus* virulence factors can be important for the development of IE, including factors that contribute to endothelial cell adhesion (table 1) (5), such as fibronectin-binding protein A and B (FnBPA and FnBPB) and von Willebrand factor binding protein (2). The ability to form biofilm, where bacteria create and adhere to an exopolysaccharide matrix, often on the surface of a medical device, can be important for the bacteria's virulence (7). These virulence factors may contribute to the high mortality rate (22 to 66%) associated with *S. aureus* IE (2). Some studies suggest that information about the genetic characteristics of the infecting strain can predict the

severity of IE, and it is of great interest to study the association between *S. aureus* genetic traits and endocarditis (8).

Whole genome sequencing (WGS) can be used to determine the genetic characteristics of bacteria. To type and find relationship between *S. aureus* strains, one can use sequence typing. This method distinguishes strains using housekeeping genes, which are essential genes and therefore seldom mutates (9). If the bacteria share identical sequences (alleles) of different housekeeping genes, they are classified as closely related. Sequence types (ST) with identical alleles for most (five or six) out of the total seven of the housekeeping genes can be grouped into the same clonal complex (CC)

Gene	Protein	Function	Reference
clfA	clfA	Adhesin, binds to human fibrinogen	(10)
clfB	clfB	Adhesin, binds to human fibrinogen	(11)
cna	cna	Adhesin, binds to collagen	(10)
fnbA	fnbA	Adhesin, binds to fibrinogen and fibronectin	(10)
fnbB	fnbB	Adhesin, binds to fibrinogen and fibronectin	(10)
isdA	isdA	Adhesin, binds to fibrinogen and fibronectin	(10)
sdrC	sdrC	Adhesin, unknown mechanism	(12)
sdrD	sdrD	Adhesin, unknown mechanism	(13)
sdrE	sdrE	Adhesin, unknown mechanism	(14)
sspA	sspA	Biofilm formation and removal	(15)
sspB	sspB	Biofilm formation and removal	(15)
vWbp	vWbp	Adhesin, binds to blood vessel wall	(16)

Table 1. Genes that encode proteins involved in adherence.

The role of fibronectin and fibrinogen

Fibrinogen and fibronectin are present in human blood and extracellular matrix, and can form coatings on indwelling medical devices. Several studies suggest that these proteins act as human ligands for *S. aureus* and that the bacteria's ability to cause infection may depend upon this interaction (17).

Research question and goals

We hypothesised that there is a difference in adhesion characteristics between *S. aureus* causing IE and carriage strains. The main objective of this project was to compare characteristics of *S. aureus* carrier strains with strains from patients with IE. We analysed the strains' ability to adhere to fibrinogen and fibronectin, the sequence types and clonal complexes of the strains and the presence of genes that encode proteins involved in bacterial adherence. We investigated whether there is an association between the ability to cause IE (*in vivo* phenotype), the bacterial adherence (*in vitro* phenotype) and the genetic characteristics.

Materials and methods

The bacterial adhesion to fibrinogen and fibronectin was evaluated using a method previously described by SJ Peacock et al. (18) and AM Edwards et al. (19). Since the method was not yet set up at the Department of Medical Microbiology, it had to be adjusted to the laboratory and the available instruments. For fixation of adherent bacteria 95% ethanol was used, as paraformaldehyde and glutaraldehyde create higher demands on ventilation and the use of personal protective equipment. Three different 96 well microtiter plates were tested (*MaxiSorp* microtiter, *Costar* microtiter plate and the *Nunclon* microtiter plate). The *MaxiSorp* microtiter plate was found to give the most stable results and was therefore used in the further experiments. To verify the method and control each run, we included a control strain with known phenotype. The method was checked for reproducibility by performing biological replicates.

Bacterial strains

The Sepsis registry of Nord-Trøndelag Health Trust contains comprehensive clinical information from all patients with positive blood cultures admitted to Levanger- and Namsos Hospitals since 1994. The registry also collects and stores the bacterial strains from each BSI episode. In this project we have included *S. aureus* strains and clinical information from 30 IE patients from this registry collected up until 2020, and 27 carriage strains collected from 2007 and 2008 from healthy people participating in a large population survey conducted in Tromsø (The Tromsø Study). The positive control was an NCTC reference strain (*S. aureus* 8325-4) given to co-supervisor Christina Gabrielsen Ås (CGÅ) by Morten Kjos, a Professor at NMBU.

Bacterial growth conditions and concentration measurements

The bacterial strains were inoculated from frozen stocks onto blood agar, incubated overnight in a *Thermo Steri-Cycle CO2 Incubator* at 37 ºC and 5% CO_2 and examined for contamination the following day. Colonies from the blood agar were then inoculated into tryptic soy broth (TSB) and incubated overnight at 37 ºC and 5% CO_2. The next day the broth cultures were centrifuged with *Eppendorf Centrifuge 5804 R* (3000xg, 10 minutes) to collect bacterial cells. The pellets were then washed three times with phosphate-buffered saline (PBS). Next, the cultures were mixed with PBS to make a bacterial suspension. Lastly, the suspensions' optic density (OD) was measured using *Shizamadzu UV Spectrophotometer* and adjusted to an OD-value of 0.90-0.94 at 650 nm, which corresponds to approximately 1 x 10^9 colony forming units (CFU) per millilitre (CFU/mL) (18).

Bacterial adherence to fibrinogen or fibronectin

A microtiter plate assay was used to assess the adherence of bacteria to solid-phase fibrinogen and fibronectin. *MaxiSorp* 96 well microtiter plates were coated with 100 µL 10 µg/mL fibrinogen (Sigma) or 100 µL 10 µg/mL fibronectin (Sigma) and incubated at 4 ºC overnight. The next day, 300 µL of 3% bovine serum albumin (BSA) in PBS was added to block the remaining sites to avoid adhesion of bacteria to non-coated areas of the well, and the plates were incubated for 1 hour at 25 ºC. The wells were then washed with 300 µL PBS three times using *Thermo Scientific Wellwash Versa*, followed by inoculation of approximately 1 x 10^8 bacteria per well and incubation at 37 ºC for 1 hour. Next, the wells were washed three times with 300 µL PBS to remove non-adherent bacteria, followed by fixation of the adherent bacteria with 200 µL ethanol (95%) for 5 minutes and a further three rounds of washing with 300 µL PBS. The fixed bacteria were then stained with 300 µL of crystal violet for 2 minutes before three new rounds of washing with 300 µL PBS. Lastly, crystal violet was solubilized with 100 µL 7% acetic acid, and the OD-value of the wells was measured at A-590 using a *FLOUstar optima* microplate reader.

Each bacterial strain was tested with five biological replicates in the same individual microtiter plate. Each microtiter plate also included *S. aureus* 8325-4 binding to wells coated with both BSA and fibrinogen or fibronectin as positive controls, and *S. aureus* 8325-4 binding to wells that were coated only with BSA as a negative control. Both the positive and the negative controls were tested in quadruplicate.

The mean absorbance value was calculated for each bacterial strain, including the negative and the positive controls. The results are reported as percentages of the mean OD value of the positive controls on the same plate after subtraction of the mean OD value of the negative controls, and referred to as adherence values.

Genome sequencing

Whole genome sequencing of the *S. aureus* strains used in this project was previously performed by the Genomic Core Facility at NTNU. Bioinformatic analyses of whole genome sequences were performed as part of a related research project. Results from genotyping as well as virulence gene prediction were used in this project to allocate the strains to sequence types and clonal complexes, and to identify the presence of 12 genes encoding proteins involved in adherence (table 1). Information on the 12 genes was obtainable for all 30 IE strains but could not be obtained for three of the 27 carriage strains. Four of the IE strains did not produce ST-results due to inadequate sequence quality, in addition to 16 of the carriage strains.

Statistical analysis

An independent-samples T test was performed to compare adherence data between the group of carriage strains and the group of IE strains. Where the results were not normally distributed, a Mann-Whitney U test was additionally performed. Fisher's exact test was used to look for overrepresentation of specific genotypes in any of the strain groups.

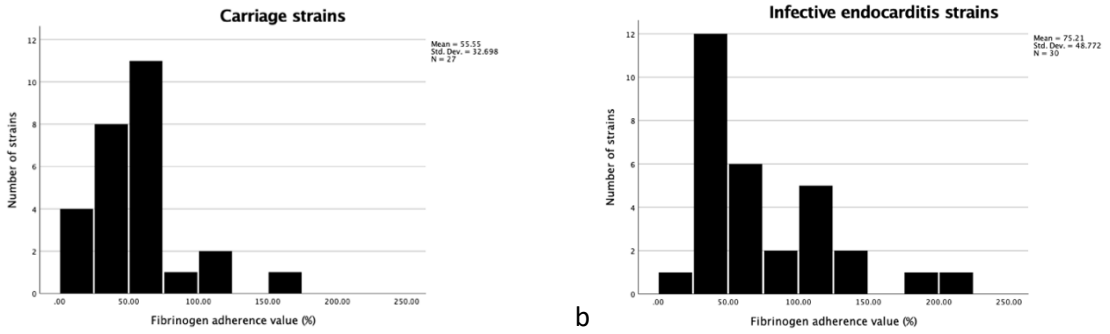
Results

Clinical description of the IE patients

Clinical information was available for 28 of the IE strains. In total, 57% of the patients were men and 43% were women. The age of the patients ranged from 23 to 93 years, with a median age of 76 years. Twenty-five patients had an underlying disease. Seventeen patients had a type of foreign object in their body, including five patients with artificial heart valves. The patient's length of stay in hospital varied from 4 to 62 days, with 13 days as the median length of stay. Thirteen patients were hospitalised for more than two weeks, while 15 patients stayed in hospital for less than two weeks. Six of the 28 patients died within 60 days of admission.

Bacterial adherence to fibrinogen

The carriage strains and the IE strains both showed variability in adherence to fibrinogen as the adherence values varied from 16% to 162% with a mean of 56% among the carriage strains (Fig. 1a), and from 21% to 220% with a mean of 75% among the IE strains (Fig. 1b). Nine and three strains had an adherence value of more than 100% among the IE strains and the carriage strains, respectively. However, the means of the two groups did not differ significantly (p=0.083). The Mann-Whitney U test showed no significant differences between the groups (p=0.117).

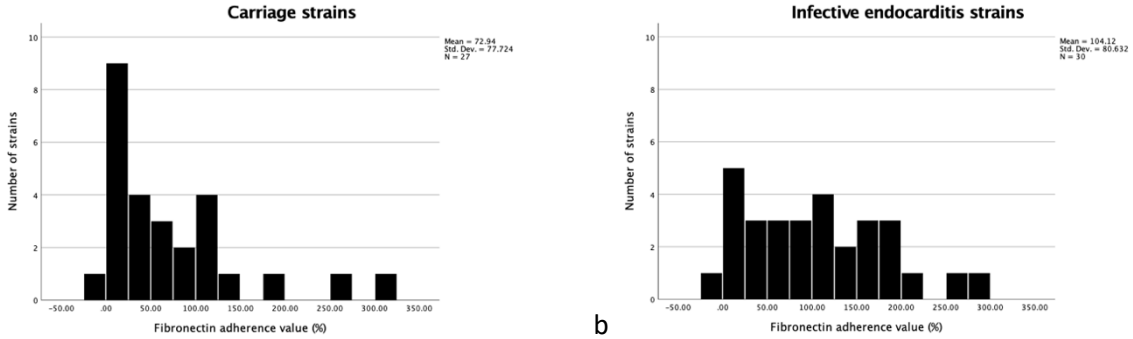


a b

Fig 1. Adherence of *Staphylococcus aureus* strains to fibrinogen from **a)** 27 asymptomatic persons (carriage strains) and **b)** 30 patients with infective endocarditis.

Bacterial adherence to fibronectin

Both the carriage strains and the IE strains showed variability in adherence to fibronectin. The adherence values ranged from –4% to 312% with a mean of 73% among the carriage strains (Fig. 2a), and from –11% to 284% with a mean of 104% among the IE strains (Fig. 2b). In the group of carriage strains, eight strains showed an adherence value of more than 100%, compared to 15 in the group of IE strains. The means of the two groups did not differ significantly (p=0.144). The Mann-Whitney U test showed no significant differences between the groups (p=0.114).



a b

Fig 2. Adherence of *Staphylococcus aureus* strains to fibronectin from **a)** 27 asymptomatic persons (carriage strains) and **b)** 30 patients with infective endocarditis.

Genetic characteristics of the strains

The 12 genes presented in table 1 were present among both the carriage strains and the IE strains (table 2). Four of these genes were significantly more common in the IE strains; *clfA*, *fnbA*, *fnbB* and *sdrC*. All stains in both the carriage group and the IE group carried the *isdA* gene.

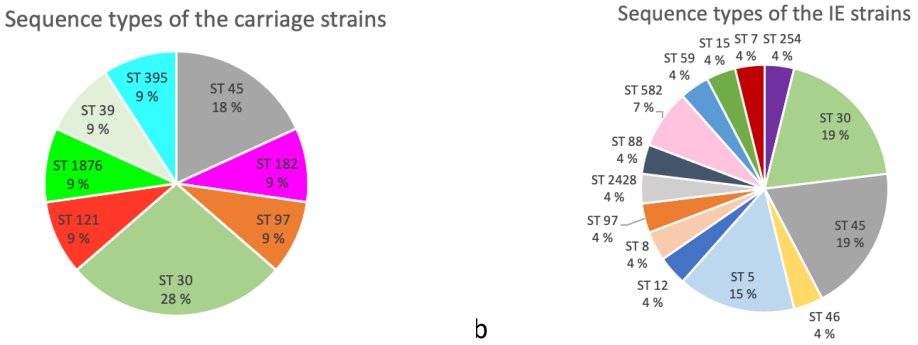
Gene	IE (n=30)	Carriage (n=24)	P-value
clfA	29 (96.7%)	9 (37.5%)	<0.001
clfB	12 (40.0%)	6 (25.0%)	0.384
fnbA	20 (66.7%)	7 (29.2%)	0.013
fnbB	21 (70.0%)	7 (29.2%)	0.006
isdA	30 (100%)	24 (100%)	
sspA	30 (100%)	23 (95.8%)	0.444
sspB	28 (93.3%)	23 (95.8%)	1.000
cna	5 (16.7%)	4 (16.7%)	1.000
sdrC	22 (73.3%)	5 (20.8%)	<0.001
sdrD	9 (30.0%)	3 (12.5%)	0.190
sdrE	20 (66.7%)	9 (37.5%)	0.054
vWbp	15 (50.0%)	14 (58.3%)	0.592

Table 2. The presence of 12 genes among 30 IE strains and 24 carriage strains, and comparison of genotypes between the two strain groups using Fisher's exact test.

Classification of strains

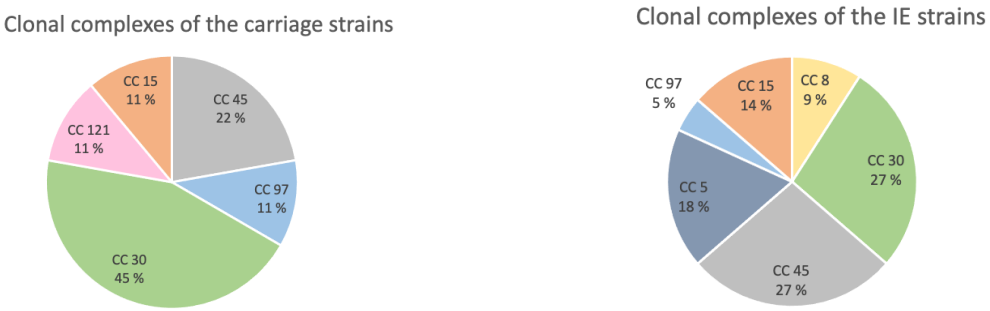
Out of the 27 carriage strains, 11 strains were assigned to eight different STs, while 16 strains could not be assigned to an ST (Fig. 3a). ST30 was most common (n=3, 28%) followed by ST45 (n= 2, 18%). Six STs were represented by only one strain. Out of the 11 strains assigned to an ST, nine were assigned to five different CCs, while two strains could not be assigned to a CC (Fig. 4a). CC30 was most common (n= 4, 45%) followed by CC45 (n= 2, 22%). Three CCs were represented by only one strain.

Out of the 30 IE strains, 26 strains were assigned to 14 different STs, while four strains could not be assigned to an ST (Fig. 3b). ST30 (n=5, 19%) and ST45 (n=5, 19%) were most common followed by ST5 (n=4, 15%). Ten STs were represented by only one strain. Out of the 26 strains assigned to an ST, 22 were assigned to six different CCs, while four could not be assigned to an CC (Fig. 4b). CC45 (n=6, 27%) and CC30 (n=6, 27%) were most common followed by CC5 (n=4, 18%). One CC was represented by only one strain.



a b

Fig 3. The sequence types (ST) of **a)** 11 carriage strains and **b)** 26 infective endocarditis (IE) strains.



a b

Fig 4. The clonal complexes (CC) of **a)** nine carriage strains and **b)** 22 infective endocarditis (IE) strains.

Discussion

The association between bacterial characteristics and the ability to cause IE

In this study, the adherence values of the carriage strains and the IE strains did not differ significantly for neither fibrinogen nor fibronectin when measured in an *in vitro* microtiter assay. This lack of correlation has previously been reported by Ythier et al. in a similar experiment (20). In contrast, an experimental endocarditis study (21) found that adherence to both fibrinogen and fibronectin plays a key role in the pathogenesis of the disease.

In the pathogenesis of IE, the bacteria must resist the shear stress of the blood stream to adhere to the vessel walls and cause infection. In a study comparing a wild-type *S. aureus* strain with three different bacterial derivates carrying mutations in global regulatory genes, *S. aureus* was found to have different adherence characteristics under flow conditions compared to static conditions (22). The four strains showed no differences in adherence to fibrinogen or fibronectin under static conditions, while the adherence of the mutant stains differed from the wild-type strain under flow. This suggests that the adhesion of *S. aureus* to fibrinogen or fibronectin is influenced

by blood flow, and that the shear stress of the blood flow could alter the surface proteins of the bacteria and affect their adherence abilities (23). Furthermore, other components of the blood, such as blood platelets, could affect the bacteria's ability to adhere *in vivo*.

As the bacterial genetic expression changes depending on external factors, bacteria with the same genotype may express different phenotypes when in different milieus. To get a realistic impression of the bacteria's adherence properties, the bacteria must be studied in an environment that is as close to real-life conditions as possible.

Previous studies have identified six *S. aureus* clonal complexes (CC1, CC5, CC8, CC15, CC30 and CC45) among individuals with IE, with CC30 being the most prevalent (24). A study conducted by Nienaber et al. also found CC30 to be more frequent in IE strains than strains from other types of *S. aureus* infections (8). Other studies suggest that there is no significant difference in the distribution of genotypes between strains from carriers compared to patients with invasive disease (25, 26).

Four out of 12 genes tested were significantly more frequent among the IE strains compared to the carriage strains. As the expression of *clfA* and *fnbA* have previously been demonstrated to be critical for the colonization of cardiac valves in IE, one would expect an overrepresentation of these genes among the IE strains (21). Peacock et al. examined the presence of *fnbA*, *clfA*, *clfB*, *cna*, *sdrC*, *sdrD* and *sdrE* in carriage strains and invasive strains (27). They found that *fnbA*, *cna* and *sdrE* were significantly more common among invasive strains compared to carriage strains. However, in another study by Lindsay et al. they did not find any overrepresentation of bacterial genes among invasive strains when examining the same genes as Peacock et al.(28).

The results from this study suggest that there is an association between some genes that code for adhesion and the ability to cause IE, but we were not able to prove that IE strains had greater adherence to fibronectin or fibrinogen *in vitro*. This could indicate that the solid phase assay is a poor predictor of *in vivo* adhesion. Our results, and the conflicting results from previous studies, could also indicate that adhesion to fibronectin and fibrinogen is not important for the development of IE. Other factors may play a more important role for development of IE, for instance human host factors, flow conditions at the cardiac valves and regulation of genes in *S. aureus* at the site of infection (20).

Strengths and limitations

A limitation of this study is that it did not directly determine the number of bacteria corresponding to each OD value of the bacterial suspension used in the adhesion assay. Instead, values from previous publications were used, which may not accurately reflect the values obtained from the instruments at the Department of Medical Microbiology.

Another limitation of this study is the small sample size, as we only analysed 30 IE strains and 27 carriage strains. The sample size became even smaller when examining the genetic characteristics of the strains, as some of the strains did not yield sufficient quality data for whole genome sequencing. It is important to note that a small sample size can increase the margin of error and potentially decrease the reliability of the results.

When reconstituting the fibronectin lyophilizate, PBS was used instead of high-quality, tissue-grade water. Due to the use of wrong reconstitution liquid, the protein did not properly dissolve, making the protein concentration used in the experiment unknown. The protein solution was agitated in an attempt to dissolve the aggregated material which may have further degraded the quality of the protein. Improper handling of the protein may be the cause of the large intrastrain variation in the OD-value of the fibronectin positive controls between the assays, as it measured from 41% to 181% of the mean OD-value of the fibronectin positive controls after subtraction of the mean OD-value of the negative controls.

In the fibrinogen assay the intrastrain variation was smaller compared to the fibronectin assay, suggesting a more stable protein concentration between the wells. A small intrastrain variation also suggests that the various steps in the method have been carried out similarly between the wells, which is a strength to this study.

Conclusion

This study found an association between IE and three genes that code for adhesins to either fibrinogen and/ or fibronectin (*clfA, fnbA* and *fnbB*) in addition to a gene (*srdC)* with unknown adhesion mechanism. Since the mean adhesion values to both fibrinogen and fibronectin in the *in vitro* adhesion assay were similar between IE and carriage strains, this *in vitro* assay may not be suitable for the assessment of *S. aureus in vivo* adhesion. Another explanation can be that the adhesion to fibrinogen or fibronectin is not a distinguishing feature of IE strains.

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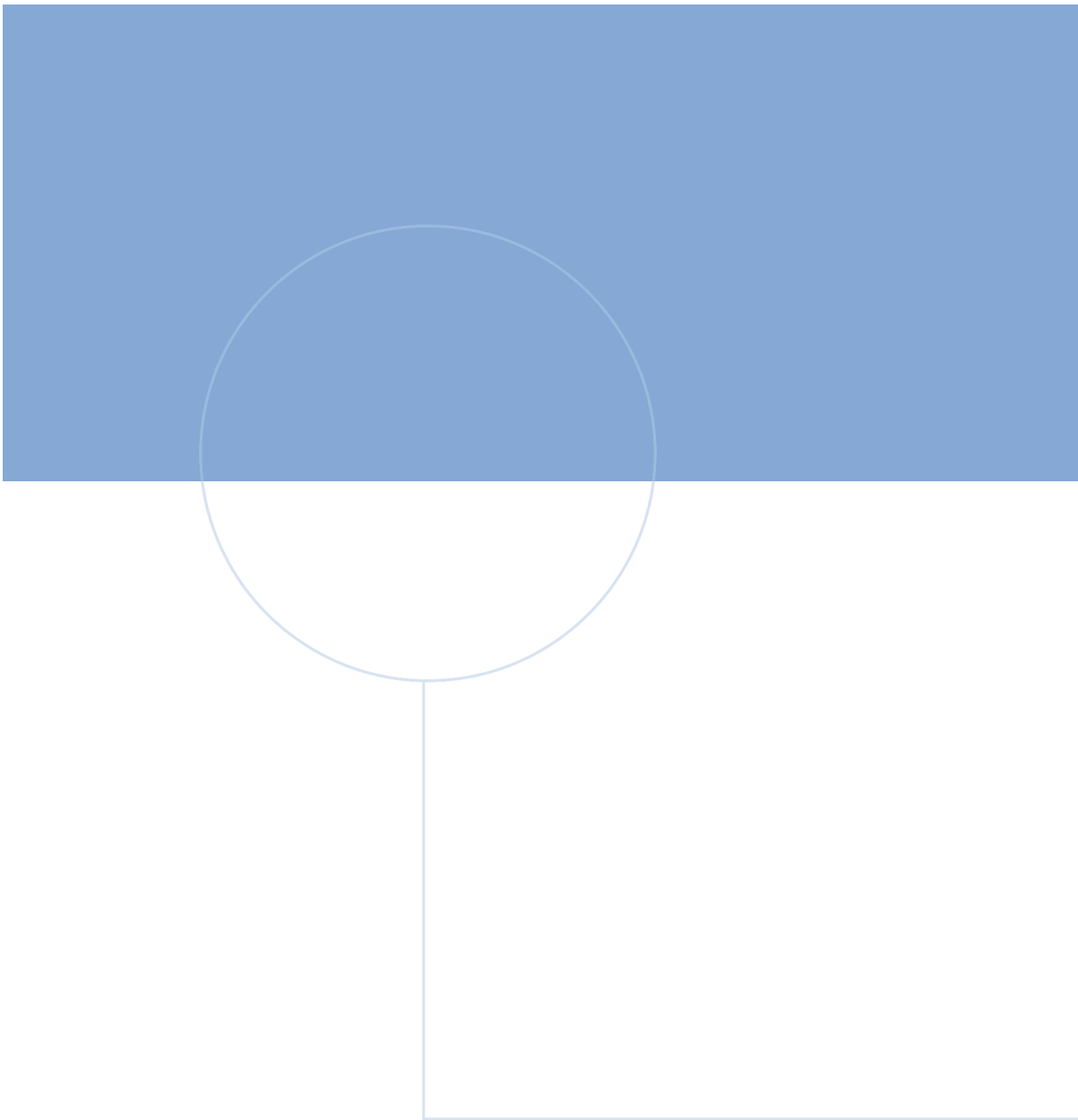

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