Biofilm Formation and Multiplex PCR detection of icaABCD Operon in Staphylococcus capitis

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Authors’ contributions

This work was carried out in collaboration among all authors. Author AAA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MFFA and SASM designed the study and analyzed the data. All authors read and approved the final manuscript.

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ABSTRACT

**Aims:** The ability to form biofilm is a major virulence factor in the virulence of the Coagulase negative Staphylococcus (CoNS) group of bacteria. Being the most predominant member of CoNS, the ability of *S. epidermidis* in causing biofilm-associated infections has been well established. On the other hand, *S. capitis* and has always been regard as a non-pathogenic species although recently it was found to be responsible in a variety of infections. Hence, this study aimed to determine the biofilm formation capabilities and the presence of icaABCD genes in clinical isolates of *S. capitis*, which have emerged as an important opportunistic pathogen in clinical settings.  

**Methodology:** *S. capitis* was isolated and identified from 17 out of 200 clinical samples. Biofilm formation assay was performed quantitatively using a microtitre plate method. Multiplex PCR primers for icaABCD genes were designed from DNA sequences coding for the icaA, B, C, and D structural genes of *S. capitis* JF930147.1 which was compared together with five other species of *Staphylococcus*. Amplification of the icaABCD genes was performed using the designated primers.  

**Results:** From the 17 strains of *S. capitis* clinical isolates, 14 were identified as *S. capitis subsp capitis* while the remaining three were identified as *S. capitis subsp ureolyticus*. Except for two of

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1. INTRODUCTION

The coagulase negative \textit{Staphylococcus} (CoNS) is a group of \textit{Staphylococcus} distinguished from the more virulent \textit{Staphylococcus aureus} by their inability to produce coagulase enzyme. This group of \textit{Staphylococcus} was considered avirulent and have long been dismissed as culture contaminants. CoNS species are known to be commensals, harmlessly living on the skin and mucous membrane of human. However, in the past few decades, the emergence of CoNS in hospital setting have become increasingly important with members such as \textit{S. epidermidis} and \textit{S. haemolyticus} being the most frequent species isolated from a variety of clinical infections [1-4].

The ability to form biofilm on biotic and abiotic surfaces is one of the virulent factors of CoNS. In clinical settings, the majority of infections by CoNS is associated with the use of foreign bodies or medical devices such as catheters, where the bacteria were able to persist on these surfaces as a layer of biofilm. For this type of infection, \textit{S. epidermidis} is the most prevalent species reported, followed by \textit{S. haemolyticus}, \textit{S. hominis} and \textit{S. saprophyticus} [5-7]. Being a predominant species of CoNS, research on the association of \textit{S. epidermidis} with medical devices has been extensively studied and serves as a role-model for other staphylococci. During the accumulation phase of biofilm formation, the main constituent of biofilm is an extracellular polysaccharide known as polysaccharide intercellular adhesion (PIA) in \textit{S. epidermidis}, or poly-N-acetyl-glucosamine (PNAG) in \textit{S. aureus}. PIA consists of glycan chain made from 1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl subunits, and has been identified as the major component responsible for intercellular adhesion of the bacteria cells to the surface, an essential factor for the virulence of \textit{S. epidermidis} [8]. PIA is synthesized by a series of enzymes encoded by the \textit{ica} operon which consists of four structural genes \textit{icaA}, \textit{icaB}, \textit{icaC} and \textit{icaD} and the regulatory gene \textit{icaR} [8,9]. The occurrence of this \textit{icaABCD} operon is most prevalent in \textit{S. epidermidis} strains associated with intravascular catheter infections. In contrast, the operon is rarely found in commensal \textit{S. epidermidis} strains isolated outside clinical settings [10]. Other protein factors involved in this accumulation phase include the accumulation associated proteins, Aap [11], the extracellular matrix binding protein (Embp) [12], extracellular DNA [13] and the biofilm-associated protein or Bap [14,15].

Biofilm formation by \textit{S. capitis}, another member of the CoNS, is less frequently reported and has not gain much interest in clinical medicine. \textit{S. capitis} has always been regarded as a non-pathogenic species and a member of the commensal flora known to colonize the human skin, especially the scalp and forehead area where sebaceous glands are abundant [16]. It can be further divided into two subspecies: \textit{S. capitis} subsp. \textit{urealyticus} and \textit{S. capitis} subsp. \textit{capitis}. Although this bacterium has been occasionally reported in some biofilm related infections which include infective endocarditis [17,18], urinary tract infection [19] and in adult bacteraemia [20], in most cases the presence of \textit{S. capitis} has been considered as a contaminant instead. However, interest on this species of CoNS has increased over the years due to the frequent incidence of \textit{S. capitis} infections especially in neonatal intensive care units, indicating the possible emergence of this bacterium as a significant opportunistic pathogen [21-26].

Since biofilm production also appears to be a virulence determinant for \textit{S. capitis}, hence the aim of this study is to identify the presence of the

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Keywords: Biofilm; \textit{icaABCD} genes; PIA, \textit{Staphylococcus capitis}.
icaABCD operon using multiplex primers which can serve as an alternative tool to determine the biofilm forming capacity of this species. The presence of these genes has also been shown in other CoNS species including *S. hominis*, *S. lugdenensis* and *S. haemolyticus* [27-30]. The demonstration of these genes could be useful as a rapid diagnosis of the severity of *S. capitis* infection and in providing an insight for the clinicians on the necessary therapy management.

The objective of this study was to determine the ability of *S. capitis* in forming biofilm on a microtiter plate, a virulence factor commonly associated with medical device-related infections. Primers for multiplex PCR were also designed to detect the presence of the icaABCD operon in each of the *S. capitis* isolates. Following this, the correlation between these two methods of biofilm assay was evaluated.

2. MATERIAL AND METHODS

2.1 Isolation of *S. capitis*

A total of 17 *S. capitis* strains were recovered from among 200 clinical samples of CoNS collected from the Pathology Department of several local hospitals. The majority of the samples were blood samples, while others include respiratory, pus, body fluid and urine samples.

Upon collection, the clinical strains were identified using standard biochemical tests and sodA gene sequence analysis to the species level as previously reported [31]. The bacteria cultures were maintained in 30% glycerol stock at -80°C. When needed, they were sub-cultured on fresh Brain Heart Infusion (BHI) agar plates at 37°C for 24 hours.

2.2 Biofilm Formation Assay

Biofilm formation assay was performed quantitatively in 96-wells flat bottom polystyrene tissue culture treated microtiter plates using the method of Stepanovic et al. [32]. Isolated colonies grown overnight on TSA plates at 37°C were inoculated into 5 ml of TSB and grown for 24 hours at 37°C under static condition. The turbidity of the cultures was then adjusted to 0.5 McFarland standard and diluted to 1:100 in TSB supplemented with 1% glucose. The experiment was done in quadruplicates whereby an aliquot of 200 ul of each strain was inoculated into four parallel wells of the microtiter plates and incubated for 24 hrs at 37°C under stationary conditions. After incubation, the contents of each well were discarded, and the wells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2) to remove planktonic bacterial cells. In between of each wash, the contents of the wells were emptied by gentle flicking and finally the plates were drained and left to dry in an inverted position. The attached bacterial cells were fixed with 150 µl methanol for 20 mins. The contents of the wells were again emptied left to dry overnight in an inverted position. Following that, the wells were stained with 150 µl 2% crystal violet for 15 mins and excess stain were then washed off by gently running under tap water before the plates were air-dried at room temperature. Finally, the remaining dye bound to the cells in each well was resolubilised by the addition of 150 µl of 95% ethanol. The microtitre plates were covered with a lid to prevent evaporation and left at room temperature for 30 mins without shaking. The absorbance of the resolubilised dye was then measured at 570 nm using a microplate reader (TECAN Infinite 200, USA). Two *S. epidermidis* strains, a non-biofilm forming ATCC 12228 (OD\(_{neg}\)) and biofilm forming ATCC 35984 (OD\(_{pos}\)) were used as negative and positive controls respectively. Each strain was tested independently with at least three replicates. Student’s t-test was used to compare the mean OD of each isolate with the mean OD of the negative control whereby a probability of P < 0.05 indicates a significant difference. Isolates with mean OD readings significantly less than the negative controls were regarded as non-biofilm formers. Isolates with significantly higher amount of biofilm than the negative control (P < 0.05) were then arbitrarily grouped into four different phenotypes, OD\(_{neg}\) < OD\(_{570} \leq 2OD_{neg}\) = weak biofilm; 2OD\(_{neg}\) < OD\(_{570} \leq 4OD_{neg}\) = moderate biofilm; 4OD\(_{neg}\) < OD\(_{570} \leq OD_{pos}\) = strong biofilm; OD\(_{570} > OD_{pos}\) = very strong biofilm formation.

2.3 Multiplex PCR Detection of the icaA, icaB, icaC and icaD Genes

The DNA sequences coding for the icaA, icaB, icaC, and D structural genes for *S. capitis* and five other common species of *Staphylococcus* were downloaded from GenBank as shown in Table 1. The sequences were aligned using ClustalW in Bioedit, and manually scanned for conserved regions. A similarity matrix and phylogenetic tree
was constructed using MEGA5 to assess the sequence divergence for the design of multiplex PCR primers that can be used to amplify the icaABCD genes from each species.

Four pairs of multiplex primers cap-ica A, B, C and D (Table 2), targeting the internal sequences of the ica structural genes were designed based on the DNA sequences of the S. capitis icaABCD operon using standard criteria. Genomic DNA extraction was performed using a commercial kit (Norgen, Canada) from culture grown overnight at 37°C, 150 rpm, in TSB. The multiplex PCR reaction was performed using HotStarTaq Master Mix kit (Qiagen) in a total volume of 25 µl. For each reaction, a volume of 12.5 µl of HotStarTaq Master Mix was used, with 1.5 µl of 1.5 ng DNA as the template, 4 µl of 10 µM of each primer and 3 µl of deionized water. The cycling condition began with initial heat activation of 95°C for 15 mins followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 65°C for 8 mins and a final extension step for 16 mins at 65°C.

3. RESULTS AND DISCUSSION

3.1 Biofilm formation by S. capitis Isolates

A total of 17 S. capitis isolates were recovered from the 200 clinical specimens. Fourteen of them were identified as S. capitis subsp capitis while the remaining three were identified as S. capitis subsp. ureolyticus [31]. Fig. 1. represents an example of the results of biofilm formation assay of S. capitis performed in a microtiter plate.

Two of the S. capitis subsp. capitis were found to be non-biofilm forming isolates while the remaining 15 or 88% were able to form biofilm, ranging from very strong to moderate biofilm formers. As shown in Fig. 2., the strongest biofilm former was isolate B102, a S. capitis subsp. capitis strain isolated from blood sample displaying a mean OD of 6.725 (P < .001) with 29.6-fold increase of biofilm material as compared to the negative control. Two other strains also produced a very strong biofilm phenotype i.e. isolate F1, a S. capitis subsp ureolyticus strain isolated from body fluid; and isolate B145, a S. capitis subsp. capitis from blood sample. F1 and B145 displayed mean OD results of 5.064 (P < .001) and 4.642 (P < .001) respectively, equivalent to 22.3- and 20.4-fold increase in biofilm thickness. In comparison, the positive control biofilm-forming strain ATCC 35984 exhibited a lower mean OD of 4.286 (P < .001) equivalent to an 18.8-fold increase of biofilm formation. A further eight isolates exhibited a strong biofilm forming ability, while four isolates exhibited a moderate biofilm phenotype. The capability to form biofilm is thus observed in both the subspecies.

| Species | Genbank Accession No. |
|---------|-----------------------|
| S. epidermidis | U43366.1 |
| S. haemolyticus | FJ472951.2 |
| S. capitis | JF930147.1 |
| S. caprae | AF246926.1 |
| S. lugdunensis | EF546621 |
| S. aureus NCTC8325 | CP000253.1 |

The DNA sequence of six Staphylococcus species downloaded from the GenBank

Table 2. Primers used for the PCR-amplification of icaABCD operon in S. capitis

| Genes | Primers | Amplicon size (bp) | Reference |
|-------|---------|-------------------|-----------|
| S. capitis icaA | cap_icaAF | GAGATGAAGCAACATCACGA | 836 | This study |
| S. capitis icaB | cap_icaBF | CCGATGAAGACCAAGAAG | 594 | This study |
| S. capitis icaC | cap_icaCF | GAATCATTATTGAGCTTTGC | 422 | This study |
| S. capitis icaD | cap_icaDF | GAGGAAATTCACCGGGA | 283 | This study |

Nucleotide sequences are written in 5’ to 3’ direction
Fig. 1. Microtiter plate assay for biofilm formation of *S. capitis*

All wells contain isolates in quadruplicates: Wells A1 to D1 = blank; A2 to D2 = negative control (*S. epidermidis* ATCC 12228); A3 to D3 = positive control (*S. epidermidis* ATCC 35984). Each of the *S. capitis* isolates were inoculated into blocks of four wells for the assay. The experiment was repeated with at least three independent replicates on different plates.

Fig. 2. Biofilm formation in *S. capitis*

Biofilm formation of *S. capitis* isolates. Error bars represent the standard error of the mean (SEM). All the isolates were able to form biofilm at different degree ranging from very strong to weak biofilm formation except for P27 and B6.7. Each experiment was repeated with at least three independent replicates.

3.2 Primers Design for *icaABCD* Operon of *S. capitis*

The presence of the *icaABCD* operon is the single genetic feature most often associated with biofilm formation amongst CoNS species [33,34]. Since the prototype *icaABCD* operon was first characterized in *S. epidermidis* [35], the presence of the operon has been reported in *S. aureus* and several species of CoNS [36-38]. Bioinformatics analysis indicates that the DNA sequences of the operon can vary substantially between different species of *Staphylococcus*. For this reason, primers design to detect the
icaA, B, C and D genes in one species may not work for the other species. Hence, in the present study, to improve the probability for detection, a new set of multiplex primers were designed for the S. capitis ica.

Multiple alignments of the ica operon DNA sequences from the six species of Staphylococcus indicates that the ica operons of S. capitis subsp capitis is 85% similar to that of S. caprae. The sequences from S. epidermidis and S. hemolyticus are highly similar at 99.4% similarity and both of this are about 76% similar to the capitis-caprae ica operon as shown in Table 3. Phylogenetic analysis in Fig 2 supports the hypothesis that the ica operons of S. epidermidis and S. hemolyticus are very closely related as both of them form a cluster, while those form S. capitis subsp capitis and S. caprae form another cluster. The S. aureus ica operon shares about 71% homology with the capitis-caprae ica, and about 70% homology with the epidermidis-hemolyticus ica while the S. lugdunensis ica operon is the most diverged within the group, and only share ~60% similarity with the other five species. The results from this analysis are similar to that reported by Fredheim et al. [36].

### 3.3 Multiplex PCR Detection of the icaA, icaB, icaC and icaD Genes in S. capitis

Using the cap-ica primers, the multiplex PCR designed to detect the presence of the icaABCD operon in S. capitis was successfully demonstrated by the amplification of the corresponding fragments as shown in Fig 3. The amplicons with the expected sizes of 836 bp, 594 bp, 422 bp and 283 bp for the icaA, B, C and D genes respectively was observed in all the isolates of both S. capitis subsp capitis and S. capitis subsp ureolyticus.

| Species             | S. epidermidis | S. haemolyticus | S. capitis | S. caprae | S. aureus NCTC8325 |
|---------------------|----------------|-----------------|------------|-----------|--------------------|
| S. epidermidis      | 99.4           |                 |            |           |                    |
| S. haemolyticus     | 76.6           | 76.4            |            |           |                    |
| S. capitis          | 75.8           | 75.9            | 84.5       |           |                    |
| S. caprae           | 69.6           | 69.5            | 71.4       | 71.0      |                    |
| S. aureus NCTC8325  | 60.8           | 60.8            | 59.8       | 59.5      | 60.0               |

*The figures represent the percentage of similarity between the six different staphylococcal species*

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**Fig. 3. Phylogenetic relationship of icaABCD operon sequences of S. capitis and five other Staphylococcus species**

*The similarity matrix and phylogenetic tree was constructed using MEGA5 to assess the sequence divergence.*
4. DISCUSSIONS

One major pathogenicity factor of CoNS is their ability to form a biofilm structure which enables the bacteria to be more resistant towards both antibiotics and host defense mechanisms [4,39]. The in vitro ability to form biofilm on polymer and other surfaces by this group of organism reflects their ability to adhere to medical devices and cause infections. This is especially significant in S. capitis infections, which are predominantly associated with in-dwelling catheters and other medical devices inserted into the body [18,21,26].

A total of 15 out of 17 or 88% of the S. capitis strains were biofilm formers strains with the majority of them exhibited either very strong or strong biofilm formation. While the ability of S. epidermidis in forming biofilm has been established, the association between biofilm formation and S. capitis infection is not well characterised. A total of 180 CoNS isolates obtained from a neonatal ICU in UK revealed that biofilm-forming S. capitis was responsible in 12 out of 29 or 41% of the infections [27]. In 2015, it was reported that a strain of clinical S. capitis AYP1020 was able to generate biofilm, although the relative production of biofilm was lower than that of S. epidermidis RP62a [40]. In contrast, several studies reported the low prevalence of biofilm formation in S. capitis. A study on coagulase negative staphylococcal bloodstream infections in neonates revealed that 15% of the 66 CoNS isolates studied were identified as S. capitis and most of these were non-biofilm formers [41]. Similarly, from 66 clinical isolates of CoNS studied, two were identified as S. capitis but none of these were able to form biofilm [28]. As the method for the quantification of biofilm production employed in these studies is similar to the current study, it is unclear of why most of the S. capitis studied failed to display any biofilm production.

In another study, a total of 60 isolates of S. capitis were obtained from blood cultures of neonates [42] whereby 52 of these were S. capitis subsp ureolyticus while the remaining eight species were S. capitis subsp capitis. Low prevalence of S. capitis subsp capitis in biofilm formation was observed whereby only one from the eight isolates was able to form biofilm while in contrast 49 or 94.2% of the S. capitis subsp
ureolyticus were biofilm formers. The difference as compared to present study could be attributed to the different method of bacterial cultivation employed. Biofilm production assessment in the study by Cui was performed using TSB supplemented with 4% NaCl while in the current study TSB was supplemented with 1% glucose. In a study on a biofilm forming S. epidermidis, it was reported that the biofilm phenotype was abolished by cultivation in growth medium containing high NaCl concentrations [43]. This is supported by another study by Fredheim [36] who suggested that biofilm formation is induced when S. haemolyticus is cultivated in TSB supplemented with glucose while a high NaCl concentration greatly reduced the level of biofilm production. Similarly, a study on biofilm production in S. lugdenensis [38] revealed that inoculation of the staphylococcal clinical isolates in TSB with NaCl would greatly reduce the biofilm production of this organism. The actual mechanism on the reduced biofilm production in the presence of NaCl however, is poorly understood.

PIA has been known to be an important component in biofilm formation in CoNS which suggests that the expression of icaABCD operon is essential to promote a biofilm-positive phenotype for this group of bacteria and plays an important role in disease pathogenesis. Several evidence supporting this perspective has been documented, both in animal models and clinical studies. A decreased in the pathogenicity of a S. epidermidis strain in animal models of foreign-body infections was observed with the inactivation of the icaA gene [44,45] while a biofilm negative-ica negative S. epidermidis strain ATCC 12228 was found to be less invasive in an animal model of catheter-related infections [46]. It has also been reported that an ica-negative strain of S. epidermidis was able to regain its ability to form biofilm when a plasmid with ica locus was transferred to the strain [47].

The use of PCR as a method for the detection of the ica operon has been widely employed as this technique is simple and reliable [48-50]. In this study, the presence of the icaABCD operon was seen in S. capitis in which all the clinical isolates were positive. This high prevalence of the ica operon in this species has also been documented in other studies. Using icaA as a marker for the icaABCD operon, 89% of S. capitis isolated from bloodstream infections in neonates were ica-positive [41]. Similarly, in another study, all eight clinical isolates identified as S. capitis subsp capitis were found to harbor the entire icaABCD operon [42].

The icaABCD operon is widespread in clinical isolates of S. epidermidis [8,34], and has been documented in many studies [51-53]. In general, it is agreed that both the icaABCD operon and the ability to form biofilm are important parameters for staphylococcal colonization and survival especially on medical devices [54]. However, the correlation between the ica gene and the ability to form biofilm particularly in S. capitis is still poorly understood and requires further investigation.

While the majority of the S. capitis isolates were strong biofilm formers, some others formed moderate to weak biofilm. One explanation of this different biofilm-forming ability among the clonal isolates could be due to the adaptation to different environmental factors. For example, in a study on S. epidermidis, it was found that most of the clinical isolates can be stimulated to produce biofilm in response to changes in the environmental encountered in the clinical setting [55]. Hence, it is likely that different environmental settings in the S. capitis isolates could be a factor in contributing to different biofilm phenotypes of the isolates.

In this study, it was also observed that two of the S. capitis isolates harbouring the icaABCD operon although they were non-biofilm formers suggesting that biofilm assessment on microtitre plate is not an accurate tool in determining the genetic capacity to produce PIA. A similar observation was reported by De Silva [27] who isolated 180 CoNS strains from a neonatal intensive care unit. The authors demonstrated that the presence of ica genes is not a definitive predictor of biofilm production as only 59 % of ica-positive CoNS strains formed biofilms on Congo red agar. It was proposed that the genetic regulation of the icaABCD operon is the factor that determines the biofilm production among the clinical isolates and not the just the presence of the icaABCD genes [56]. The most likely explanation is that for some strains, additional factors present in the human body may be required to trigger biofilm production, in the presence of a complete icaABCD operon. In addition, the possibility of the genes being over-expressed or under-expressed, resulting in different ability in biofilm-forming cannot be ruled out. Another possibility which could have occurred in the non-biofilm forming isolates is point mutation in the icaABCD operon followed
by subsequent transcription of non-functional proteins. Hence, the failure to detect the operon in the two isolates suggests that the biofilm formation and assessment on microtitre plates is not a definitive tool in determining the production of PIA. Instead, genetic regulation of the icaABCD operon could be influenced by environmental factors that facilitated biofilm production when exposed to the human system.

5. CONCLUSIONS

The majority of S. capitis strains isolated from clinical samples were able to form biofilm in vitro, suggesting that this ability may be a major factor in S. capitis opportunistic infections through indwelling medical devices. Multiplex PCR was successfully developed to detect the presence of all the icaABCD genes in S. capitis, demonstrated by the amplification of the corresponding fragments. Isolates which carry the icaABCD operon but were non-biofilm forming may carry mutations that could not be detected by PCR alone, in which case it is suggested that future studies should include DNA sequencing of the operon which may allow for better characterization.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The research was approved by the Research Ethics Committee, Universiti Teknologi MARA, Malaysia (REC 600-IRMI (5/1/6))

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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