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ORIGINAL ARTICLE

Comparison between *ica* Operon Expression and Biofilm Formation in Methicillin-resistant *Staphylococcus Aureus* Isolated from Central Venous Catheters under Different Environmental Conditions

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Methicillin-resistant *Staphylococcus aureus* (MRSA), Biofilm, Indwelling medical devices, Intercellular adhesion (*ica*) operon

ABSTRACT

Background: *Methicillin-resistant Staphylococcus aureus (MRSA) infections are complicated by the ability of the organism to grow in surface-adhered biofilms on a multitude of inert synthetic surfaces including those involving indwelling medical devices. Intensive care unit (ICU) patients using central venous catheters (CVCs) are particularly at risk of acquiring device-related infections, which involve biofilms.* ***Objectives:*** *This study was carried out to compare intercellular adhesion (ica) operon expression and biofilm formation in MRSA isolated from CVCs grown under different environmental conditions.* ***Methodology:*** *Seven hundred sixteen central venous catheters tips were tested for MRSA colonization. Semiquantitative measurements of biofilm formation were determined for all MRSA isolates grown under different environmental conditions: Brain heart infusion (BHI) medium, BHI supplemented with 4% sodium chloride (NaCl) and BHI supplemented with 1% glucose (Glu). The ica operon expression were compared in all MRSA isolates grown under different environmental conditions using RT-PCR.* ***Results:*** *The overall catheter tip colonization rate was 36.87%. Staphylococci were isolated from 56.06%. The distribution of the isolated Staphylococci was as follow: Staphylococcus epidermidis (S. epidermidis) 34.8%, Staphylococcus aureus (S. aureus) 12.12% and other Coagulase negative Staphylococci CoNS 9.09%. Out of 32 S.aureus isolates 9 were MRSA (28.125%). Under standard laboratory conditions in BHI medium 22.22% of MRSA isolates were capable of biofilm development. This number increases to 77.77% when grown in BHI supplemented with 1% glucose. In contrast, growth in BHI supplemented with 4% NaCl induces biofilm in 11.11%. Among the 9 MRSA isolates, growth in the presence of NaCl resulted in activation of ica transcription in 8 strains but failed to induce substantial biofilm development in any of these isolates [weak -but measurable- biofilm formation was detected in medium supplemented with NaCl by one strain]. Glucose-mediated induction of biofilm formation in the 9 MRSA isolates correlated with weakly to moderately increased ica operon expression in 6 isolates. Interestingly, ica operon transcription was more potently activated by NaCl than by glucose in all of the MRSA isolates examined except one strain.* ***Conclusion:*** *There appears to be little correlation between ica operon regulation and biofilm formation in MRSA, suggesting that the ica operon and polysaccharide intercellular adhesin, or poly-N-acetylglucosamine (PIA/PNAG) may not be required for biofilm development in MRSA.*

INTRODUCTION

S*taphylococci are* the leading cause of biofilm-associated infections, such as intravascular catheter-related sepsis and infective endocarditis, which are associated with unacceptably high morbidity, mortality and costs[1]. Two key factors have been linked with suboptimal outcomes in treating such invasive *S. aureus* infections: (i) the organism's abilities to develop resistance to multiple antibiotics [e.g., MRSA, vancomycin [VAN]-intermediate *S. aureus* (VISA) and VAN-resistant *S. aureus* (VRSA)], and (ii) its ability to form biofilms on both native tissues and implanted biomaterials. It is well known that *S. aureus* cells within a complex biofilm matrix are refractory to both systemic antimicrobial agents and host immune responses[2]. This makes medical treatment of these infections very

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difficult, and often the implanted device has to be removed or replaced[3].

Analysis of the factors that assemble cells into a biofilm has revealed the occurrence of strains that produce either a PIA/PNAG exopolysaccharide- or a protein-dependent biofilm [4].

Production of a polysaccharide adhesion by *ica* operon-encoded enzymes is currently the best-understood mechanism of staphylococcal biofilm development. Although the majority of clinical *S. aureus* isolates contain the *ica* operon, the expression of the *ica* operon and biofilm production are tightly regulated under in vitro conditions. In the laboratory, CO2 levels, anaerobicity, glucose, and osmotic stress can all influence *ica* operon expression and/or biofilm development [5].

Evidence that biofilms can develop on intravascular devices, including CVCs, has been well documented. Colonization of the outer lumen of the catheter by microorganisms is usually the result of the catheter's proximity to skin flora. Colonization of the inner lumen of catheters may be the result of a break in aseptic handling of the device prior to insertion or of the exposure of the end connectors to water, soil, or contaminated intravenous fluids. Both Gram-positive and Gram-negative bacteria have been isolated from biofilms on CVCs [6].

This work aimed to compare *ica* operon expression and biofilm formation in MRSA isolated from CVCs grown in BHI medium alone, BHI supplemented with 4% NaCl and BHI supplemented with 1% glucose.

METHODOLOGY

Bacterial isolates.

Seven hundred sixteen CVCs tips were collected from 600 patients in ICU of Surgery department Alahli Hospital (it is a private 250-bed tertiary general hospital in Doha, Qatar) from October 2011 to November 2013. Each catheter tip was transferred to the Microbiology and clinical Immunology laboratory (Qatar Armed Forces hospital) in tubes containing 10 ml of brain heart infusion broth.

Sonication of each tube was performed for 1 min (at 55,000 Hz and 125 W), and vortexing for 15 s., 0.1 ml of the sonicated broth was streaked onto sheep blood agar plates. The plates were incubated aerobically for 48 h at 37°C. Staphylococcal strains were identified by their characteristic growth morphologies, Gram stain characteristics and Vitek 2 GP cards (bioMe′rieux, Marcy I′Etoile, France) (according to the manufacturer's instructions).

Catheter colonization.

Catheter colonization was defined as a positive semiquantitative tip culture by sonication (≥100 CFU/plate) [7].

Susceptibility testing with Vitek2 system.

Vitek 2 AST-P580 cards (cefoxitin concentration 6μg/ml; oxacillin concentrations 0.5, 1 and 2 μg/ml) were inoculated according to the manufacturer's instructions. For each isolate, a Vitek 2 card was inoculated on same day from a single culture plate with bacterial suspension equivalent to a 0.5 McFarland standard. Readings were automatically taken every15 min. The instruments' Advanced Expert System (AES) interprets any *S. aureus* isolates that tests positive by the cefoxitin screen (MIC ≥ 6 μg/ml) and or oxacillin MIC ≥ 4 μg/ml as oxacillin resistant.

All MRSA were subjected to the following steps:

Biofilm assays.

Semiquantitative measurements of biofilm formation were determined with Nunclon tissue culture-treated (Surface) 96-well polystyrene plates (Nunc, Denmark), based on the method of **Christensen et al.**[8], with the following modification. Each MRSA isolate was grown in individual wells of 96-well plates at 37°C in BHI medium alone, BHI supplemented with 4% NaCl and BHI supplemented with 1% glucose. After 24 h of growth, the plates were washed three times with distilled water to remove unattached bacteria and dried for 1 hour at 60°C, as recommended by **Gelosia et al.**[9], prior to staining with a 0.4% crystal violet solution. Each strain was tested three times, and average results are presented. A biofilm-positive phenotype was defined as an optical density at 492 nm of ≥ 0.17[10].

RNA purification and RT-PCR.

RT-PCR was done using RNA prepared from cultures grown at 37°C to an *A*600 of 2.0 to standardize RNA loading between samples. Bacterial cells were collected and immediately stored in RNA*later* (Ambion) to ensure maintenance of RNA integrity prior to purification. Total RNA was subsequently isolated using the GenElute Total RNA purification kit (Sigma) according to the manufacturer's instructions following a 5- to 10-min pretreatment of the cells with 50 μg of lysostaphin in 100 μl of 50 mM EDTA. Purified RNA was eluted and stored in RNA*secure* resuspension solution (Ambion). Residual DNA present in RNA preparations following purification was removed using DNA*free* DNase treatment and removal reagents (Ambion).

RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) following the manufacturer's recommended protocol. Master mixes were prepared using primers as follows: for *gyrB* transcripts (nt 219 to 536), 5′-TTATGGTGCTGGGCAAATACA3′ and 5′-CACCATGTAAACCACCAGATA3′[11]; for *icaA* transcripts, 5′-TCTCTTGCAGGAGCAATCAA3′ was used as the forward primer (primer 1, corresponding to nucleotides 1337 to 1356), and 5′-TCAGGCACTAACATCCAGCA3′ was used as the reverse primer (primer 2, corresponding to nucleotides 1505 to 1524). The two primers include a 188-bp

region[12]. For all of these RT-PCRs, RT was performed at 55°C for 30 min followed by 23 amplification cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 20 s. The *gyrB* gene is constitutively expressed in *S. aureus* and was used as an internal standard in these experiments[13].

Analysis of RT-PCR data.

Aliquots of the amplified products were analyzed on a 3% agarose gel. Densitometry was performed using the Stratagene Eaglesight software package to compare relative expression levels between samples.

RESULTS

In this work 716 CVCs were collected from 600 patients. The overall catheter tip colonization rate was 36.87% (264/716 CVCs). *Staphylococci* were isolated from 56.06% (148/264 colonized CVCs).

The distribution of the isolated *Staphylococci* was as follow*: S.epidermidis* 34.8% (92/264), *S. aureus* 12.12% (32/264) and other CoNS 9.09% (24/264).

Out of 32 *S. aureus* isolates 9 were MRSA (28.125%)

Under standard laboratory conditions in BHI medium 2/9 MRSA isolates (22.22%) are capable of biofilm development. This number increases to 7 (77.77%) when grown in BHI supplemented with 1% glucose. In contrast, growth in BHI supplemented with 4% NaCl induce biofilm in one strain (11.11%) (Fig. 1).

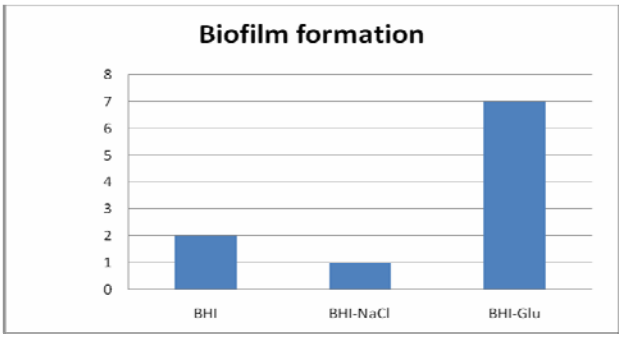

Fig.1: Biofilm formation of MRSA under different environmental conditions

By RT-PCR, *ica* operon transcription was detected in all MRSA isolates (Fig. 2).

Among the 9 MRSA isolates, growth in the presence of NaCl resulted in activation of *ica* transcription in 8 strains but failed to induce substantial biofilm development in any of these isolates [weak -but measurable- biofilm formation was detected in medium supplemented with NaCl by one strain]. Glucose-mediated induction of biofilm formation in the 9 MRSA isolates correlated with weakly to moderately increased *ica* operon expression in 6 isolates. Interestingly, *ica* operon transcription was more potently activated by NaCl than by glucose in all of the MRSA isolates examined except one strain (Fig. 2).

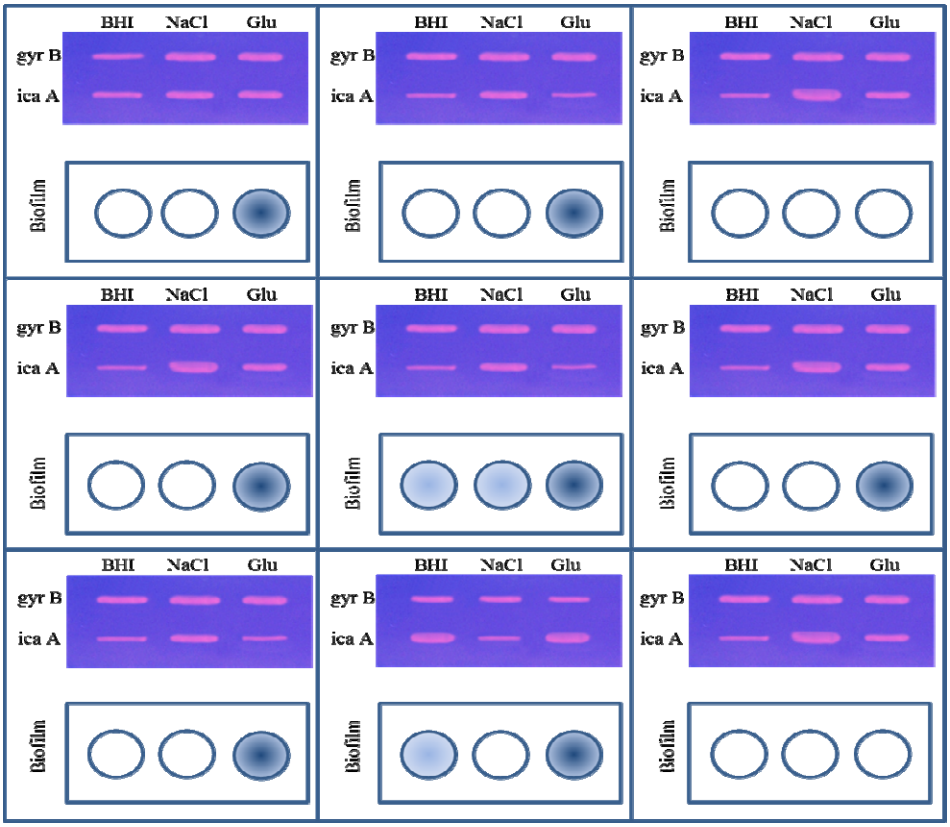

Fig. 2: Comparison of *ica* operon expression and biofilm regulation in 9 MRSA clinical isolates grown in BHI medium or in BHI supplemented with 4% NaCl or 1% glucose.

DISCUSSION

Among staphylococci, production of a polysaccharide adhesin, termed PIA or PNAG, by *ica* operon-encoded enzymes is currently the best-understood mechanism of biofilm development. Although the majority of clinical *S. aureus* isolates contain the *ica* operon[12], expression of the *ica* operon and biofilm production are tightly regulated under in vitro conditions [10].

This work aimed to compare *ica* operon expression and biofilm formation in MRSA isolated from CVCs grown in BHI medium alone and BHI supplemented with 4% NaCl or 1% glucose.

In this study 716 CVCs were collected from 600 patients. The overall catheter tip colonization rate was 36.87% (264/716 CVCs). *Staphylococci* were isolated from 56.06% (148/264 colonized CVCs). The distribution of the isolated *Staphylococci* was as follow*: S. epidermidis* 34.8% (92/264), *S. aureus* 12.12% (32/264) and other CoNS 9.09% (24/264). Similarly, **Guembe et al.**[7] documented an overall catheter tip colonization rate of 26.2% in a study done in Spain, with *Staphylococci* isolated from 56.25% of them. *S. epidermidis, S. aureus and* other CoNS accounted for 35.4, 12.5 and 8.3% respectively of their total isolates. **Maki**[14] noted that CVCs pose a greater risk of device-related infection than does any other indwelling medical device. Higher colonization rate was documented in USA by **Donlan et al.**[15] who examined needless connectors attached to CVCs and documented 63% colonization rate.

In this work the prevalence of MRSA among *S. aureus* isolates was 28.125% during the period (2011-2013). In a study done by **El-mahdy et al.**[16]**,** in Doha Qatar during the period (2009-2010) they documented 21% of MRSA among their *S. aureus* isolates. Lower results (15%) were documented by **Khalaf et al.** [17] who conduct their study during the period (2008-2010) in the same country. We notice an increase of MRSA isolates in Doha Qatar.

High rates of MRSA in Middle Eastern and North African countries have been reported in some national studies from Tunisia, Jordan, Lebanon and Saudi Arabia[18;19;20; 21]. In a study done in Egypt by **Borg et al.**[22] they documented that more than 50% of the *S. aureus* blood cultures isolates were methicillin-resistant. In a study done by **Chua et al.**[23] they documented 24% of MRSA from CVCs in USA.

In this study under standard laboratory conditions in BHI medium 2/9 MRSA isolates (22.22%) are capable of biofilm development. This number increases to 7 (77.77%) when grown in BHI supplemented with1% glucose. In contrast, growth in BHI supplemented with NaCl induce biofilm in one strain (11.11%). In a study done by **O'Neill et al.**[10] 8% for their MRSA isolates were able to form biofilm in BHI medium alone, 74% with 1% glucose supplement and only 3% with 4% NaCl. In a study done by **Seidl et al.**[24] they documented that biofilm formation in *S. aureus* under in vitro growth conditions is generally promoted by high concentrations of sugar.

In this work among the 9 MRSA isolates, growth in the presence of NaCl resulted in activation of *ica* transcription in 8 strains but failed to induce substantial biofilm development in any of these isolates [weak -but measurable- biofilm formation was detected in medium supplemented with NaCl by one strain]. Glucose-mediated induction of biofilm formation in the 9 MRSA isolates correlated with weakly to moderately increased *ica* operon expression in 6 isolates. Interestingly, *ica* operon transcription was more potently activated by NaCl than by glucose in all of the MRSA isolates examined except one strain

Rachid et al.[25] studied the effect of glucose and NaCl on biofilm development and *ica* transcription in MRSA. Biofilm development was significantly induced by glucose. In contrast, NaCl was insignificantly induce biofilm formation in MRSA isolates. They documented that biofilm development in MRSA isolates which is primarily glucose induced, is ica independent. Their experiments suggest the involvement of a protein adhesin in glucose-induced MRSA biofilm development.

In a study done by **Fitzpatrick et al.**[5]**,** they found that Biofilm formation was increased four- to eightfold in all their MRSA isolates when grown in BHI medium supplemented with glucose compared to BHI alone. In contrast, growth in BHI supplemented with NaCl failed to induce biofilm in these isolates. **Fitzpatrick et al.**[26] reported the existence of a possible correlation between methicillin susceptibility and the environmental regulation of biofilm development in clinical *S. aureus* isolates and that icaADBC-independent biofilm formation was possible in clinical MRSA isolates.

Seidl et al.[24] studied the effect of glucose on biofilm formation in *S.aureus*. Biofilm formation was shown to be promoted by adding increasing concentrations of glucose. Without glucose supplementation, no significant biofilm formation was observed irrespective of the fact that BHI already contained substantial amounts of glucose. In contrast to the findings of **Beenken et al.**[27]**,** who observed biofilm formation of *S. aureus* in polystyrene microtiter plates only in media that were supplemented with both NaCl and glucose and when the wells of the microtiter plates were precoated with plasma proteins, neither addition of salt to the growth medium nor precoating the microtiter plates with plasma proteins was essential for, or increased biofilm formation in **Seidl et al.**[24] experiments, suggesting that strains with altered

adhesion/biofilm-forming capacities may exist in different laboratories.

Evidence for different mechanisms of biofilm development in MRSA isolates is likely to be significant in our understanding of the pathogenesis of MRSA device-related infections involving biofilms and for the development of novel therapeutics targeting this important staphylococcal phenotype.

CONCLUSION

There appears to be little correlation between *ica* operon regulation and biofilm formation in MRSA, suggesting that the *ica* operon and PIA/PNAG may not be required for biofilm development in MRSA.

These data raise the question of why the *ica* locus is maintained, expressed, and regulated in MRSA isolates. Perhaps the acquisition of resistance to multiple antibiotics that target the cell wall accidently results in impaired biosynthesis or export of PIA/PNAG. Such changes in the cell surface of MRSA strains may also be accompanied by the unintended redeployment of a cell surface protein(s) not normally involved in adherence to inanimate surfaces on naked (as opposed to surfaces coated with host proteins) polystyrene or other biomaterials. Given that many *S. aureus* cell wall-anchored proteins are involved in binding to host matrix proteins, the interesting possibility exists that in the absence of PIA/ PNAG, one or more of these adhesins may also play a role in biofilm development in MRSA.

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