Anti-Biofilm Activity of Sub-Inhibitory Povidone-Iodine Concentrations against *Staphylococcus Epidermidis* and *Staphylococcus Aureus*

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Received 11 July 2009; accepted 8 January 2010
Published online 24 February 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.21110

ABSTRACT: Biomaterial-related infections continue to hamper the success of reconstructive and arthroplasty procedures in orthopaedic surgery. Staphylococci are the most common etiologic agents, with biofilm formation representing a major virulence factor. Biofilms increase bacterial resistance to antimicrobial agents and host immune responses. In staphylococci, production of polysaccharide intercellular adhesin (PIA) by the enzyme products of the *icaADBC* operon is the best understood mechanism of biofilm development, making the *ica* genes a potential target for biofilm inhibitors. In this study we report that the antibacterial agent povidone-iodine (PI) also has anti-biofilm activity against *Staphylococcus epidermidis* and *Staphylococcus aureus* at sub-inhibitory concentrations ($p < 0.001$). Inhibition of biofilm by PI correlated with decreased transcription of the *icaADBC* operon, which in turn correlated with activation of the *icaR* transcriptional repressor in *Staphylococcus epidermidis*. These data reveal an additional therapeutic benefit of PI and suggest that studies to evaluate suitability of PI as biomaterial coating agent to reduce device-related infections are merited. © 2010 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:1252–1256, 2010

Keywords: biofilm; biomaterial related infection; povidone-iodine; *Staphylococci*

Despite improvements in orthopaedic surgery over the last three decades, deep infections continue to hamper the success of reconstructive and arthroplasty procedures. Prosthetic joint infection is associated with significant morbidity and sometimes mortality of the individual. It also represents a tremendous economic burden to healthcare institutions and the general public as a whole.[1–3]

Recent analysis in the United States suggests that the incidence of deep infection in total hip arthroplasty will exceed 50% by 2030 with similar incidence occurring earlier for knee arthroplasty procedures.[2]

More than half of these prosthesis-associated infections are caused by *S. epidermidis* and *S. aureus*[4,5] with biofilm formation representing a major step in their pathogenesis. Biofilm offers protective barrier to organisms, resulting in resistance to antimicrobial agents[6,7]and host immune responses.[8–10] Biofilm production and accumulation is mediated by polysaccharide intercellular adhesin (PIA)[11]which is encoded by the *icaADBC* operon.[12] The operon is composed of biosynthetic genes (*icaADBC*) and the divergently transcribed *icaR* gene, which encodes a transcriptional repressor of the *ica* operon in *S. epidermidis*[13] and *S. aureus*.[14] Environmental stress factors[15–17] and sub-inhibitory concentration of some antibiotics[18] have been identified to trigger staphylococcal biofilm formation through increased *icaADBC* expression. Likewise alcoholic skin disinfectants have been shown to increase *icaADBC* mediated biofilm formation in *S. epidermidis*.[15] Sub-inhibitory concentrations of the commonly used disinfectants chlorhexidine and benzalkonium-induced biofilm development by *S. epidermidis* strain CIP53124 strain.[19] In contrast, mild oxidative stress, induced by sub- inhibitory concentrations of hydrogen peroxide has been found to significantly reduce biofilm formation in *S. epidermidis* through down regulation of *icaADBC* operon expression.[20]

Staphylococcus epidermidis is a skin commensal. This organism can exist on skin surface as micro-colonies that share similar properties of biofilms in terms of resistance to biocides.[21,22] Surgical site infection from colonising skin flora leads to deep infection. During elective surgery, prosthetic joint infection is most likely to occur during insertion of the implant through the skin. Skin disinfectants that promote biofilm growth at sub-inhibitory concentration may contribute to occurrence of biomaterial-related infection.[23]

Povidone-iodine (PI) is a complex of polyvinyl pyrrilidine and triiodine ions that is widely used as an antiseptic in trauma and orthopaedic surgery. Sub-inhibitory concentrations of PI may exist clinically via dilution of the antiseptic by tissue fluids and blood in operative wounds. Sub-lethal dose of PI may also occur during dilution for deep wound irrigation in trauma or wound debridement. The practice of pre-operative "skin shower" with dilute PI may leave residual sub-inhibitory concentrations of the antiseptic on the skin. Because of the widespread use of PI, we investigated the possible impact of sub-inhibitory concentrations of this antiseptic on biofilm development by *S. epidermidis* and *S. aureus*. We determined the minimum inhibitory concentration (MIC) of clinically used PI (betadine) and investigated the effect of sub-lethal PI doses on *icaADBC* transcription and biofilm development in *S. epidermidis* and *S. aureus*.

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MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

The well-characterized biofilm-forming reference strains *S. epidermidis* 1457[24] and *S. aureus* RN4220[25] were grown at 37°C on Brain–Heart Infusion (BHI) (Oxoid, UK) medium supplemented as indicated with PI (Standardized Betadine® Antiseptic solution USP 10% (w/v) 100 mg of PI per 1 ml of solution, Seton Healthcare Group plc, England).

Determination of Minimum Inhibitory Concentration (MIC) of PI

S. epidermidis 1457 and *S. aureus* RN4220 were grown for 24 h at 37°C in BHI and BHI supplemented with a range of PI concentrations from 0.01% to 10% (w/v). Determinations of MIC and minimum bacteriocidal concentration (MBC) were performed as previously described.[19,20]

Biofilm Assays

Semi-quantitative determinations of biofilm formation in 96-well tissue culture plates (Nunc, Denmark) using bacteria grown at 37°C in BHI, and BHI supplemented with 0.17%, 0.35%, or 0.7% of PI were performed as described previously.[26–28] After overnight incubation at 37°C, plates were washed three times with distilled H_2O and dried for 1 h at 56°C prior to staining adherent biofilm with a 0.4% crystal violet solution for 15 min. The excess stain was removed by rinsing with distilled water and the absorbance of the adhered, stained cells was measured at A_{492} using using 96 well plate readers (SpectraMax2 Spectrophotometer, Molecular Devices Corp., Sunnyvale, CA). Experiments were repeated eight times and average data are presented.

RNA Purification and Analysis

For RNA isolation, bacterial cells were collected at mid logarithmic phase of the growth curve ($A_{600} = 2$) and immediately stored in RNA*later* (Ambion, Austin, TX) at −20°C to ensure maintenance of RNA integrity prior to purification. Total RNA was subsequently isolated using TRIzol®, Sigma-Aldrich, St. Louis, MO, Max Bacterial Reagent kit (Invitrogen, UK) according to the manufacturer's protocol following a 5–10 min pre-treatment of the cell with 50 μg of lysostaphin (Ambion) in 100 μl of 50 mM EDTA. Purified RNA was eluted and stored in RNA*secure* resuspension solution (Ambion), and the integrity of the RNA was confirmed by agarose (1%) gel electrophoresis. Residual DNA present in the RNA preparations following purification was removed using DNA*free* DNase treatment and removal agents (Ambion). RNA quantity and purity was determined spectrophotometrically.

Real-Time Quantitative PCR

The oligonucleotide primers used to amplify *gyrB*, *icaA*, and *icaR* mRNA transcripts were previously described.[13] All primers were supplied by MWG-Biotech (Ebersberg, Germany). In *S. epidermidis* 1457, *gyrB* transcripts, 5′-TTATGGTGCTG-GACAGATACA-3′ and 5′-CACCGTGAAGACCGCCAGATA-3′; for *icaA* transcripts, 5′-AACAAGTTGAAGGCATCTCC-3′ and 5′-GATGCTTGTTTGATTCCCT-3′; for *icaR* transcripts, KCR1 5′-GGTAAAGTCCGTCAATGGAA-3′ and KCR2 5′- CGCAAT-AACCTTATTTTCCG-3′. For *S. aureus* RN4220 *gyrB* transcripts, SAGYR1 5-TTA TGG TGC TGG ACA GAT ACA-3 and SAGYR2, 5-CAC CGT GAA GAC CGC CAG ATA-3; for *icaA* transcripts, STAA1 5′-GAG GTA AAG CCA ACG CAC TC-3′ and STAA2 5′-TGG GTA TTC CCT CTG TCT GG-3′; for *icaR* transcripts, STAAR1 5′-TTC TCA ATA TCG ATT TGT ATT GTC AAC-3′ and STAAR2 5′-TGT CAG GCT TCT TGT TCA ATG -3′.

Amplification, detection and analysis of mRNA were performed using a Rotor-gene instrument (Rotor-Gene, Corbett Life Science, Sudney, Australia) with QuantiFast SYBR Green RT-PCR kit (Qiagen, UK) following the manufacturer's recommended protocol. The reaction mixture (20 μl) contained 10 μl of 2x QuantiFast SYBR Green RT-PCR Master Mix, 1 μl (25 pmol) each of the appropriate forward and reverse primers, 0.2 μl of QuantiFast RT Mix, 6.7–7.3 μl RNase-free water, and 90 ng of template RNA from above. Six additional samples per experiment had no template RNA in order to verify each primer pair.

For Rotor-Gene RT-PCRs, reverse transcription was performed at 50°C for 10 min, followed by initial PCR activation step of 95°C for 5 min. This was followed by a 35 amplification cycle of two-step cycling of denaturation at 95°C for 10 s, and a combined annealing/extension at 60°C for 30 s. Dissociation curves revealed no non-specific products in any amplification reaction. Data from the Rotor Gene detection instrument were analysed with Rotor-Gene software version 6 (Corbett Life Science) according to the comparative CT (threshold cycle) method.[29–31] The constitutively expressed *gyrB* gene was used as an internal control.[13,32] *gyrB* expression was measured in parallel with measurements of *icaA* and *icaR* transcript levels and used to standardize variations in RNA loading between samples as described previously.[13] The final results were expressed as fold increase or decrease in *icaA* or *icaR* gene expression relative to *gyrB*.

Statistical Analysis

Statistical analysis using ANOVA was performed to compare differences in biofilm forming capacity by *S. epidermidis* 1457 and *S. aureus* RN4220 grown in BHI and BHI supplemented with different concentrations of PI. Correlation between biofilm formation and concentration of PI was analysed using Spearman rank order correlation. Comparison of significance in gene expression was assessed by the equal-variance Student's *t*-test. A *p*-value below 0.05 was considered significant in all cases.

RESULTS

Impact of PI on Growth, Viability and Biofilm Forming Capacity of *S. epidermidis* and *S. aureus*

The MIC of PI (Betadine) was 1.4% (w/v) (data not shown). Based on these data, we examined the impact of sub-inhibitory concentrations of serial twofold dilutions of PI (0.17%, 0.35%, and 0.7%) on staphylococcal biofilm development. In this way we were able to ensure that differences in biofilm forming capacity in medium supplemented with PI could not be attributed to differences in growth (Fig. 1A and B). Semiquantitative 96 well plate biofilm assays revealed that sub-inhibitory concentrations of PI significantly inhibited biofilm formation by *S. epidermidis* 1457 ($p < 0.001$) and *S. aureus* RN4220 ($p < 0.001$) (Fig. 1A and B). Therefore, in addition to its known antibacterial activity, PI also significantly inhibits staphylococcal biofilm formation at sub-inhibitory concentrations.

Impaired *S. epidermidis* 1457 and *S. aureus* RN4220 Biofilm Development in Sub-Inhibitory Concentrations of PI is Associated With Repression of the *icaADBC* Biofilm Gene Cluster

To examine if reduced biofilm forming capacity in the presence of PI was related to altered expression of the

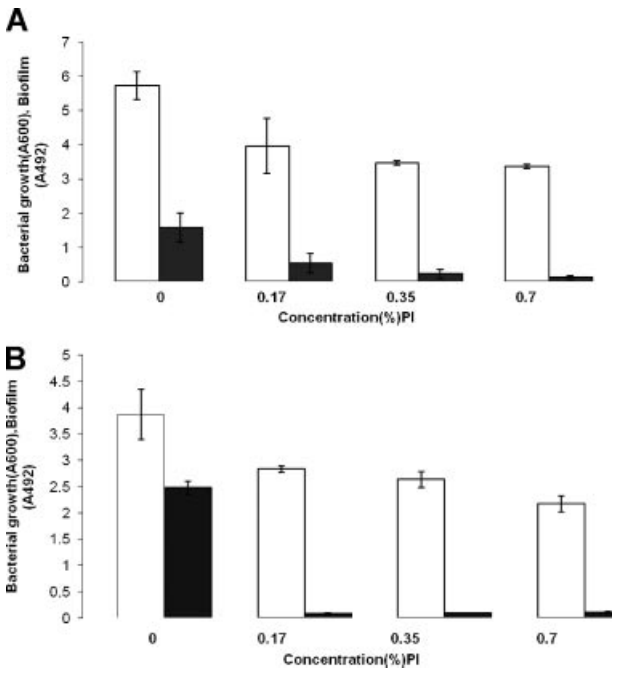

Figure 1. Comparison of *S. epidermidis* 1457 (A) and *S. aureus* RN4220 (B) growth (white) and biofilm (black) in BHI broth (control 0%) and BHI supplemented with 0.17%, 0.35%, and 0.7% PI. Growth was measured at A_{600} using a cell density meter (Ultra spec 10, Amersham Biosciences). Biofilm assays (A_{492}) were performed in 96-well microtitre plates. Experiments were repeated at least eight times and standard deviations are indicated.

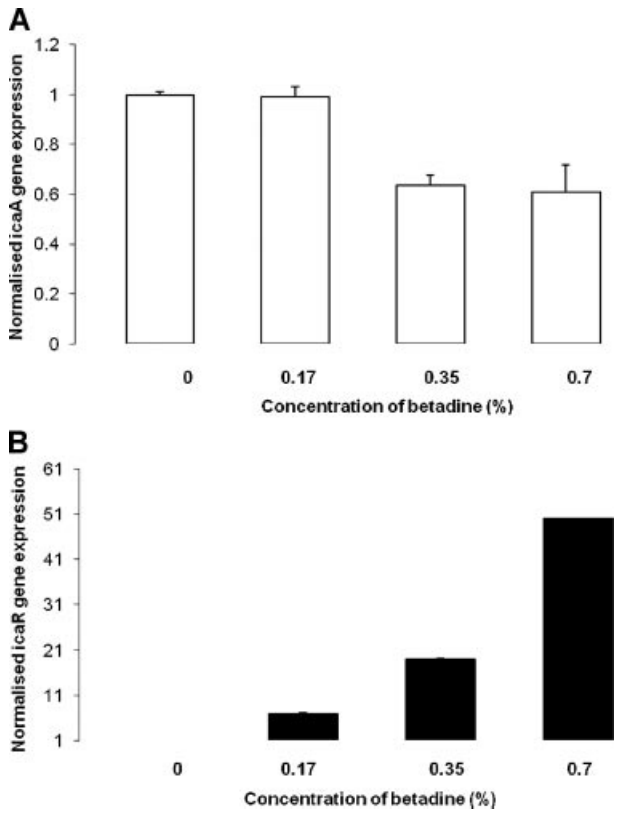

Figure 2. Comparative measurement of *gyrB* (control), *icaA* and *icaR* transcription in *S. epidermidis* 1457. Total RNA was prepared from cultures grown to $A_{600} = 2.0$ at 37°C in BHI and BHI supplemented with 0.17%, 0.35%, and 0.7% PI. (A) *S. epidermidis* 1457 *icaA* gene expression at different PI concentrations. (B) *S. epidermidis* 1457 *icaR* gene expression at different PI concentrations. Experiments were repeated at least three times and standard deviations are indicated.

icaADBC locus, real-time RT-PCR was used to measure levels of the *icaA* mRNA transcripts. Consistent with the reduction in biofilm, *icaA* transcription was also reduced in sub-inhibitory concentrations of PI (Figs. 2A and 3A).

To investigate the genetic basis for decreased levels of *icaA* expression, quantitative real-time RT-PCR was used to measure levels of *icaR*, which encodes a repressor controlling *icaADBC* operon transcription.[13] There were 7-fold (0.17%, $p < 0.005$), 19-fold (0.35%, $p < 0.027$), and 50-fold (0.7%, $p < 0.0003$) increases in *icaR* gene expression at PI concentrations of 0.17%, 0.35%, and 0.7% respectively in *S. epidermidis* (Fig. 2B). There was therefore an inverse relationship between the levels of *icaA* and *icaR* expression, which correlated with biofilm forming capacity. However the dramatic activation of *icaR* expression at sub-inhibitory PI concentrations was associated with relatively modest decreases in *icaADBC* transcription suggesting that PI can influence biofilm via *icaR*-dependent and *icaR*-independent pathways. Taken together these data indicate that sub-inhibitory concentrations of PI negatively affect biofilm development in *S. epidermidis*, in part through increased *icaR* expression and decreased transcription of the *icaADBC* biofilm locus. In contrast, in *S. aureus* RN4220, decreased *icaA* transcription in the presence of PI was not associated with altered expression of *icaR* indicating that PI influences expression of the *ica* locus in *S. epidermidis* and *S. aureus* in different ways (Fig. 3B).

DISCUSSION

The increase in drug-resistant bacterial strains and the challenges in developing antimicrobial drugs to specifically target bacterial biofilm production are major issues in the development of new therapeutic strategies for biomaterial related infections. In vitro studies have shown that bacterial cells within biofilms are resistant to antibiotic concentrations 500–1,000 times higher than those that will kill their planktonic (free-floating) counterparts.[33–36] Current strategies under investigation for controlling bacterial biofilms include inhibition of microbial quorum-sensing system,[37] enhancement of antimicrobial penetration with use of bioacoustics[38] and bioelectric effects,[39–42] and by modulation of biofilm-promoting genes[43–45] Staphylococcal PIA expression and biofilm formation is preceded by *icaADBC* transcription[46] which in turn is controlled by environmental factors.

Interestingly, at a concentration of 1.4%, PI inhibited growth of *S. epidermidis* and *S. aureus* reference strains and isolates from prosthetic joint infections (data not shown). The clinical in-use dose of PI (10% betadine) has been shown to be cytotoxic to human primordial cells.[47,48] PI inhibits DNA, RNA, and protein synthesis in cells, even if the treatment causes no reduction of cell

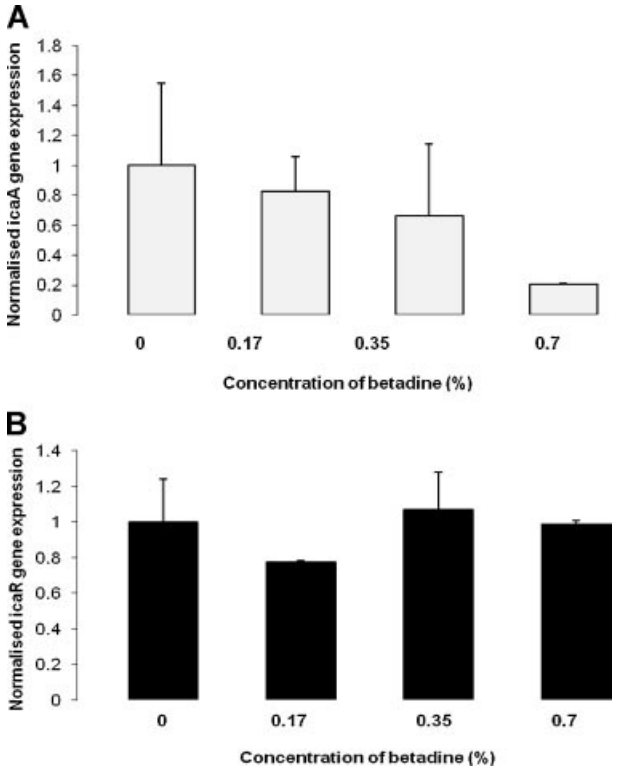

Figure 3. Comparative measurement of *gyrB* (control), *icaA* and *icaR* transcription in *S. aureus* RN4220. Total RNA was prepared from cultures grown to $A_{600} = 2.0$ at 37°C in BHI and BHI supplemented with 0.17%, 0.35%, and 0.7% PI. (A) *S. aureus* RN4220 *icaA* gene expression at different PI concentrations. (B) *S. aureus* RN4220 *icaR* gene expression at different PI concentrations. Experiments were repeated at least three times and standard deviations are indicated.

survival.[49] A prospective, randomized study revealed that a 3.5% PI solution prevented post operative wound infection after spinal surgery in all of the 208 patients in the study group.[50] The current clinical-in use concentration may therefore need to be reviewed.

Our study revealed that sub-inhibitory concentrations of PI (betadine) inhibited *S. epidermidis* and *S. aureus* biofilm development. Our findings also identified a genetic basis for impaired biofilm formation at sub-inhibitory PI concentrations. Specifically exposure of *S. epidermidis* 1457 to PI was associated with decreased *icaADBC* expression and a concomitant increase in *icaR* transcription. These data are consistent with the role of *icaR* as a transcriptional repressor of the *ica* operon.[13,20] However the dramatic activation of *icaR* transcription in various PI concentrations was associated with a relatively modest decrease in *icaADBC* transcription indicating that PI can also influence *ica* operon expression and biofilm independently of *icaR*. Growth of *S. epidermidis* in H_2O_2 has a smilar effect to PI[20] whereas exposure to 4% ethanol has the opposite effect leading to *icaR* repression and activation of *icaADBC*.[13] We believe further studies to evaluate the suitability of PI as biomaterial coating agents are merited. In contrast to *S. epidermidis*, growth of *S. aureus* in medium supplemented with PI was not associated with altered *icaR* expression even though *ica* operon transcription was significantly repressed. Interestingly *icaR* regulation is already known to be different in *S. aureus* and *S. epidermidis*.[51] For example, the stress-induced sigma factor σ^B controls *icaR* transcription in *S. epidermidis* but not *S. aureus*.

In summary, our data reveal that in addition to its known antibacterial properties, sub-inhibitory concentrations of PI can also inhibit *S. epidermidis* and *S. aureus* biofilm development at least in part by repressing transcription of *icaADBC*. However the mechanism of action of PI-mediated biofilm inhibition differs between *S. epidermidis* and *S. aureus*.

ACKNOWLEDGMENTS

The authors thank Dr. Peter Doran and all the staff of UCD Clinical Research Center, for their supports. No financial support was received from internal source or any other organization for this research work.

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