ORIGINAL ARTICLE

Titanium Kirschner Wires Resist Biofilms Better Than Stainless Steel and Hydroxyapatite-coated Wires: An *In Vitro* Study

James P McEvoy[1], Philip Martin[2], Arshad Khaleel[3], Shobana Dissanayeke[4]

ABSTRACT

Aim: External fixation surgery is frequently complicated by percutaneous pin site infection focused on the surface of the fixator pin. The primary aim of this study was to compare biofilm growth of clinically isolated pin site bacteria on Kirschner wires of different materials.

Materials and methods: Two commonly infecting species, *Staphylococcus epidermidis* and *Proteus mirabilis*, were isolated from patients' pin sites. A stirred batch bioreactor was used to grow these bacteria as single culture and co-cultured biofilms on Kirschner wires made of three different materials: stainless steel, hydroxyapatite-coated steel and titanium alloy.

Results: We found that the surface density of viable cells within these biofilms was 3x higher on stainless steel and 4.5x higher on hydroxyapatite-coated wires than on the titanium wires.

Conclusion: Our results suggest that the lower rates of clinical pin site infection seen with titanium Kirschner wires are due to, at least in part, titanium's better bacterial biofilm resistance.

Clinical significance: Our results are consistent with clinical studies which have found that pin site infection rates are reduced by the use of titanium relative to stainless steel or hydroxyapatite-coated pins.

Keywords: Bacterial adhesion, Biofilms, External fixation, Infection, Orthopedics.

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INTRODUCTION

External fixation is used widely to treat bone fractures and as a technique in limb reconstruction.[1] Metal pins known as Kirschner wires, or K-wires, are implanted into the bone above and below a fracture and an external frame is attached, fixing the bone segments in place to allow effective bone union.[2] External fixation is used for traumatic fractures which is, in itself, a leading cause of disease burden worldwide.[3] The number of external fixation procedures is increasing in many developed countries, coincident with an aging population and a rise in the frequency of fractures in geriatric patients.[4]

Pin sites (also known as "pin tracks") are prone to infection.[5] The infection rate varies widely between studies; up to 100% in some animal models[6] and clinical studies,[7] with the majority of published clinical estimates being closer to 50%.[8] Infection rates remain high even with antibiotic prophylaxis and regular pin site cleaning with topical antiseptics[9] and are further exacerbated by comorbidities such as diabetes.[10] Pin site infections are treated in the first instance with systemic antibiotics and in the last resort by removing the infected pins.[9] Chronic infection may lead to pin loosening and accompanying loss of bone alignment and, in rare cases, to osteomyelitis and bacteraemia.[7]

Pin site bacteria are part of the commensal skin flora which become opportunistic pathogens within the wound.[11] *Staphylococci* are most commonly implicated, with *S. aureus* and *S. epidermis* accounting for the majority of infections.[7,12] Gram-negative bacteria such as *Escherichia coli, P. mirabilis*, and *Pseudomonas aeruginosa* are also found commonly.[13]

Bacterial biofilms on the surface of pins act as the focus of infection.[14] Biofilms form when free-floating, planktonic bacteria attach to solid surfaces using flagella or fimbriae.[15] Adhesion is followed by bacterial growth and secretion of exopolymeric matrix substances, mostly polysaccharides, which stick bacterial cells to one another and to the colonized surface.[15] These strongly surface-associated communities allow bacteria to survive both the host immune system and clinical interventions such as antibiotic treatment.[11] The ease with which a particular bacterial strain forms a biofilm depends on the material surface; the physical characteristics (e.g., roughness) and chemical nature (e.g., hydrophobicity/hydrophilicity) have both been found to determine susceptibility to biofilm growth.[16]

Following improvements in perioperative sterility and postoperative pin site care,[17] one strategy for reducing infection rates further is to use pins which are biofilm resistant or otherwise antimicrobial.[18] The pin materials in common use are titanium alloys and stainless steel, with or without a hydroxyapatite coating.[18] Most

[1,2,4]Department of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK

[3]Rowley Bristow Orthopaedic Unit, Ashford and St Peter's Hospitals NHS Foundation Trust, Chertsey, Surrey, UK

Corresponding Author: James P McEvoy, Department of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK, Phone: +44 7759236692, e-mail: james.mcevoy@rhul.ac.uk

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clinical studies have found titanium pins are less often infected than uncoated stainless steel pins,[19–21] although some researchers have found no significant difference.[5] In animal models, some studies have detected lower infection rates with titanium than with stainless steel[22] and in others a small or nonsignificant difference.[23] *In vitro* studies on bacterial adhesion are ambiguous, with studies mostly on staphylococcal species failing to show a consistent preference for either material.[24]

Hydroxyapatite-coated pins have been shown unequivocally to improve bone contact through osseointegration which, in turn, reduces the frequency of pin loosening in both humans and animals.[25] It is unclear, however, whether the improved bone contact made by hydroxyapatite-coated pins reduces clinical infection rates,[26] despite an *in vitro* study showing decreased staphylococcal adherence to such pins.[27] The interpretation of *in vitro* studies in this area has been complicated by variation in important factors such as the bacterial species and strains selected, biofilm definition and measurement, alloy composition, and the surface treatment of the metal.

In this study, clinically interpretable *in vitro* biofilm data were obtained by first isolating and identifying clinical pin site bacteria and then growing selected clinical strains as biofilms on as-received, commercially available K-wires made from titanium alloy, uncoated stainless steel, and hydroxyapatite-coated steel. A bioreactor was used to process samples in parallel, ensuring identical biofilm growth conditions for an accurate comparison of viable (and potentially pathogenic) cell density. The use of patient-derived bacterial strains and untreated commercially available K-wires provides added practical relevance on pin site infection. The study's main finding that titanium resists biofilm growth better than the other two helps to explain the superior clinical outcomes reported for this metal.

MATERIALS AND METHODS

Clinical Bacteria Collection

Bacteria were isolated from pin site swabs taken from patients with lower limb external fixation devices at St. Peter's Hospital, Surrey, UK. A swab of each pin site at the clinically uninfected wire or wound interface (4–5 per patient) was taken from three patients and transported in Amies transport medium to the laboratory. The swabs were then transferred into phosphate-buffered saline (PBS, pH 7.5) and incubated overnight at 37.5°C. Each swab was then removed from its solution, and the overnight cultures were mixed with glycerol (150 µL/mL). These glycerol stocks were stored at −80°C until required.

Clinical Bacteria Isolation

Bacteria were isolated on three types of agars (Oxoid): eosin methyl blue agar, mannitol salt agar, and sheep blood agar. Clinically isolated culture stocks were plated onto each type of agar, and distinct colonies were assessed by gram staining and light microscopy. Distinction criteria were based on colony form, margin, elevation, differing hemolysis, mannitol fermentation, and type of agar. Isolated colonies were incubated in liquid growth medium overnight, then mixed with glycerol (150 µL/mL), and stored at −80°C until required.

Extraction and Amplification of Bacterial DNA

DNA was extracted from the bacterial isolates using standard techniques[28] and amplified with nested polymerase chain reaction (nPCR).[29] The nPCR products were assessed using ethidium bromide in a 1.8% agarose gel subjected to electrophoresis at 100 V, 200 mA, 100 W for 45 minutes. Amplicons of approximately 700 bp [outer primer (OP)] and 300 bp [inner primer (IP)] were expected and compared against a 100-bp ladder (New England Biolabs: Hertfordshire, UK). The nPCR products were sequenced (Eurofins: Wolverhampton, UK), and the nucleotide sequences were analyzed using the online nucleotide basic local alignment search tool, BLAST.[30]

Microtiter Plate Assay

A microtiter plate assay was used to measure the biofilm-forming ability of each clinical isolate.[31] Five microliters of overnight culture was pipetted into 1 mL lysogeny broth (LB) in 24-well plates. Cultures were incubated at 37.5°C for 16 hours with agitation at 160 rpm and incubated for a further 24 hours without agitation to allow biofilms to settle. The growth medium was pipetted out and the wells washed three times with PBS to remove loose cells that were not attached to the biofilm. Wells were stained with 1% crystal violet solution for 5 minutes and washed three times with PBS to remove any residual dye. Two milliliters of 95% ethanol was used to solubilize the stain and absorbance was read at 600 nm on a SpectraMax 190 plate reader (Molecular Devices: Berkshire, UK) to measure biofilm growth in each well. All isolates were grown in parallel with a strong biofilm former (*E. coli* Nissle 1917) and a weak one (*E. coli* DH10B) as a positive and negative control, respectively.

Bioreactor Biofilm Growth

A stirred-tank batch bioreactor (Fig. 1), adapted for use with 1.8-mm orthopedic K-wires, was built in-house. Its design was based on that of the CDC Biofilm Reactor (Biosurface Technologies Corporation: Bozeman, Montana, USA)[32] and was built from high-density polyethylene, polypropylene, and polycarbonate to allow for autoclaving between batches. Growth media was stirred and maintained at 37.5°C (±2°C) using a heated magnetic stir plate (VWR: Leicestershire, UK) set at 180 rpm. A pH electrode and meter (Mettler Toledo: Leicester, UK) were used to continuously monitor the temperature and pH of the growth medium, which was either 10% diluted LB broth (Sigma-Aldrich: Dorset, UK) or human serum (TCS Biosciences, Bucks, UK). Biofilms were grown on orthopedic wires inserted into the bioreactor for 48 hours from the point of inoculation of the growth medium. A waste pipe/nutrient replacement system allowed users to remove waste products and to replace nutrients in a sterile manner. The insertion of K-wires and nutrients and the removal of wastes took place in a class II biosafety cabinet under sterile conditions.

Kirschner Wires

Uncoated 316L stainless steel K-wires were obtained from De Soutter Medical (Bucks, UK), Ti-6Al-4V titanium alloy K-wires were obtained from JPP Management (Scionzier, France) and hydroxyapatite-coated steel K-wires were obtained from Ortho Solutions (Essex, UK). Wires were autoclaved before they were positioned in the bioreactor.

Fluorescent Microscopy of Biofilms

Biofilms for visualization were grown in the bioreactor on 22 mm × 70 mm glass coverslips, immersed in LB growth medium, and held in place by a custom-built coverslip holder. After 48 hours of biofilm growth, the coverslips were removed, washed in PBS, and fixed in

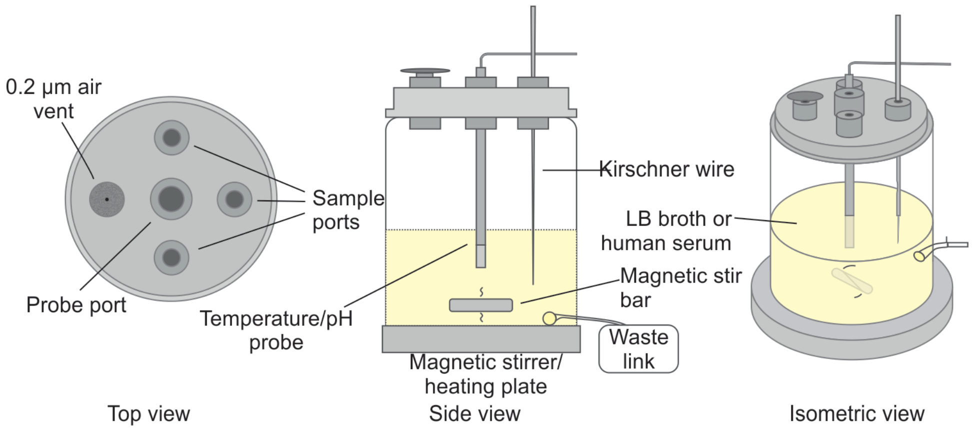


Fig. 1: Schematic diagram of the stirred batch tank bioreactor used to grow biofilms on orthopedic K-wires

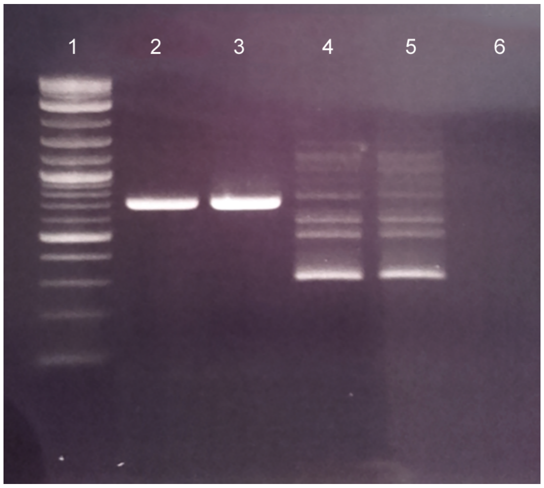


Fig. 2: Agarose gel electrophoresis of the nPCR products. (1) 100-bp ladder; (2) OP with *S. epidermidis* DNA; (3) OP with *P. mirabilis* DNA; (4) IP with *S. epidermidis* DNA; (5) IP with *P. mirabilis* DNA; and (6) Negative control

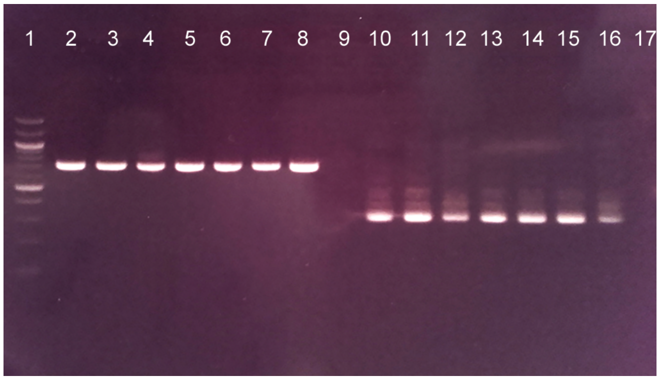


Fig. 3: Agarose gel electrophoresis of the nPCR products by species. (1) 100-bp ladder; (2) OP *S. epidermidis*; (3) OP *S. epidermidis*; (4) OP *S. epidermidis*; (5) OP *S. aureus*; (6) OP *S. aureus*; (7) OP *P. mirabilis*; (8) OP *P. mirabilis*; (9) OP negative control; (10) IP *S. epidermidis*; (11) IP *S. epidermidis*; (12) IP *S. epidermidis*; (13) IP *S. aureus*; (14) IP *S. aureus*; (15) IP *P. mirabilis*; (16) IP *P. mirabilis*; and (17) IP negative control

95% methanol for 10 minutes. Fixed slides were then stained using Invitrogen filmtracer® SPYRO ruby biofilm matrix stain (Thermo Fisher Scientific: Hertfordshire, UK) according to the manufacturer's instructions. Coverslips were attached to a glass slide using glue with white tack (UHU) around the edges of the coverslip to elevate it and preserve the three-dimensional structure of the biofilm. A Nikon Eclipse Ti-E fluorescent microscope with a mercury lamp set at 450 nm was used to excite the stain and visualize the biofilms.

Comparison of Biofilm Formation on Orthopedic Materials

Biofilms were grown inside the bioreactor (Fig. 1) on 1.8-mm-diameter K-wires in 10% LB diluted with PBS. This medium was replaced by undiluted human serum for some experiments. To efficiently detach the biofilms from the K-wires,[33] 4 mL PBS was pipetted into a 20 mL test tube, and the biofilms were dispersed into the PBS from the surface of the K-wires by ultrasound treatment. The K-wires were then immersed in 1% crystal violet solution for 10 minutes and examined under 55× magnification to confirm the complete removal of the biofilm from the pin. Sonicated hydroxyapatite-coated K-wires were checked under 55× magnification without staining because the crystal violet stained the coating, making any biofilms present indistinguishable from the K-wire. Loss of the hydroxyapatite coating during sonication was addressed by cutting the end of the wire after each round of sonication to produce a fresh surface for the next experiment.

A drop plate method was adapted to assess the number of viable cells.[34] The surface density of viable cells in the biofilms was calculated using the formula: viable cell surface density (CFU/cm^2) = $\log_{10}$ [(mean colony count/drop volume) (10^{dilution}) (PBS volume/SA)], where SA = colonized surface area of the K-wire. The surface area was calculated in turn using the formula $SA = 2\pi rd + \pi r^2$, where d = immersion depth (cm) and r = K-wire radius (cm).

Statistical Methods

SPSS Statistics 21.0 (IBM: Armonk, New York, USA) was used to analyze the microbiological results which were represented graphically as mean values ± standard error. One-way analysis of variance tests were used to evaluate the difference between groups.

RESULTS

Bacterial Identification

The nPCR products were examined by agarose gel electrophoresis (Figs 2 and 3). Amplicons of ~709 and ~287 bp in size were observed, equating to the OP and IP, respectively. The PCR products were, therefore, of the expected size, and no DNA contamination was

Table 1: BLAST results of bacterial isolates

Closest species match	*E-value*	*Identity (%)*	*Number of nucleotides*	*Sequence*
S. aureus	2^{-130}	100	257	ATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGCAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGA
S. epidermidis	5^{-131}	99	265	ATCTTGACATCCTCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACTTACAAT
P. mirabilis	8^{-134}	100	272	TTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTGATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGATCAATCTC

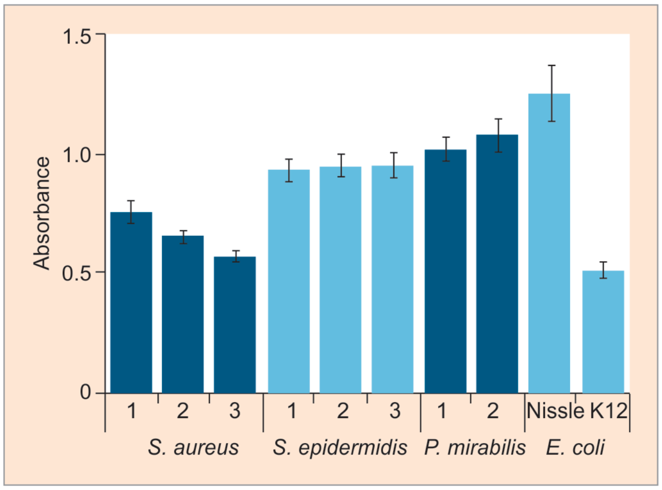


Fig. 4: Microtiter plate assay of biofilm formed by isolates, compared with controls. Absorbance of the crystal violet stain was measured at 600 nm. The columns represent the means of the four repeats with error bars representing ± standard error

observed in the negative controls. Species identity was assigned to gel lanes once sequencing has been completed.

The BLAST results of the nPCR products are shown in Table 1. Three species commonly associated with wound infections were identified: *S. aureus*, *S. epidermidis*, and *P. mirabilis*. All sequences exhibited *E*-values far below the confidence threshold of 10^{-5}, indicating a very low probability of a random match.

Microtiter Plate Assay of Biofilm Growth

Figure 4 shows the results of the microtiter assay from each clinical isolate; each assay comprised four repeats per 24-well plate, and the assay was repeated four times. All of the clinical isolates grew as biofilms on the plates, and *P. mirabilis* and *S. epidermidis* formed biofilms almost as well as the positive control *E. coli* Nissle 1917. These two species were taken forward for further investigation. The three *S. aureus* isolates showed less biofilm growth than *S. epidermidis*, in line with previous results.[35]

Fluorescent Microscopy of Biofilms

Figure 5 shows epifluorescent microscopic images of co-cultured biofilms of *P. mirabilis* and *S. epidermidis* on glass coverslips. Both spherical *S. epidermidis* (green circles) and swarming rod-shaped *P. mirabilis* (yellow circles) are visible within the same biofilm. The heterogeneous structure typical of bacterial biofilms is apparent, with clusters of cells separated by large voids in which no cells are present.[36]

Comparison of Biofilm Growth on K-wires

Biofilms of patient-isolated *S. epidermidis* and *P. mirabilis*, both separately and in co-culture, were grown in the bioreactor on K-wires made from different materials. Figure 6 shows that titanium alloy showed less biofilm formation than stainless steel and hydroxyapatite-coated steel under all conditions tested. Titanium's relative resistance to biofilm growth across all our experiments (Fig. 6D) equates to a 4.5× decrease in biofilm growth on titanium relative to hydroxyapatite and a 3.0× decrease relative to stainless steel. *P. mirabilis* biofilms (Fig. 6B) grew slightly better than *S. epidermidis* biofilms (Fig. 6A), a result which is consistent with our microtiter plate assay. Biofilms in human serum (Fig. 6C) grew better than in diluted LB medium (Figs 6A and B). Co-cultured biofilms in human serum (Fig. 6C) grew no better than biofilms of *P. mirabilis* alone (data not shown).

DISCUSSION

Bacterial biofilm formation on the surface of orthopedic pins allows infections to develop and persist.[37] Appropriately chosen pin materials could, therefore, be used to prevent biofilm formation and thereby reduce infection rates.[18] *In vivo* clinical studies are clearly required to compare surgical infection rates and to inform practice. Such experiments, however, cannot easily reveal the contribution of biofilm growth to infection because the developing biofilms are hidden under the patient's skin, and the biofilms are likely to be disturbed as the pins are removed.[38] By using a physiologically relevant *in vitro* system, we were able to compare the growth of clinically isolated bacterial biofilms on commercially available K-wires of different materials.

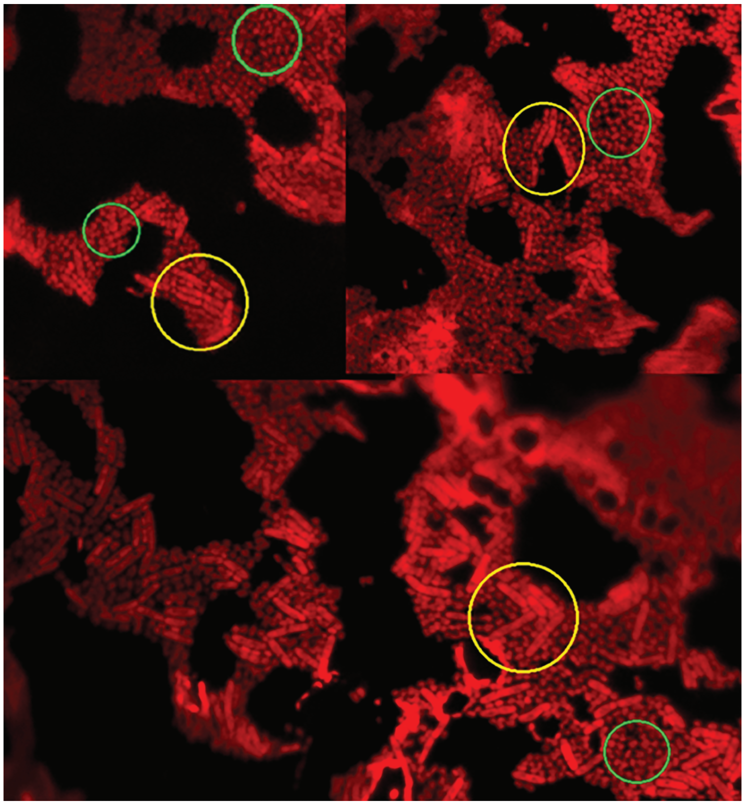


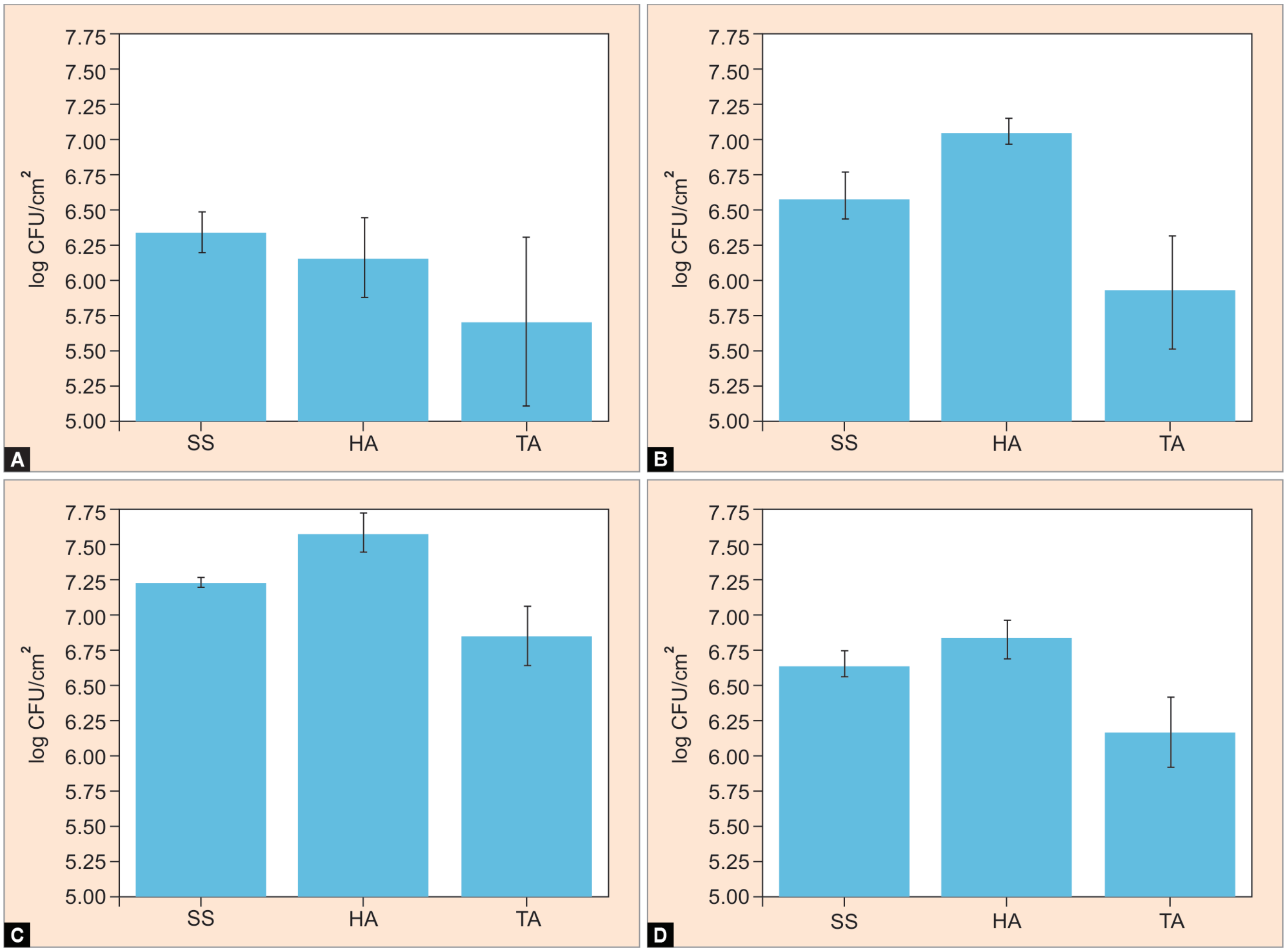
Fig. 5: Epifluorescent 100× images of co-cultured biofilms of *S. epidermidis* (green circles) and swarming *P. mirabilis* (yellow circles), grown and imaged on glass coverslips

This study found that *S. epidermidis* and *P. mirabilis*, two common pin site bacteria, developed biofilms on titanium alloy K-wires with a surface density of viable cells 3.0× less than on uncoated stainless steel. There is evidence that titanium pins lead to better clinical outcomes than stainless steel pins.[19–21] Our results suggest that one of the reasons for this commonly observed superiority might be the greater resistance to bacterial biofilm growth on titanium and thus to lower infection rates.

Titanium has been found previously in some studies to resist bacterial adhesion better than stainless steel[23,39] although other researchers found minimal differences.[24,35] The mechanisms that underlie the low susceptibility to bacterial adhesion and biofilm growth on titanium might be from a smoother nanostructure and formation of a thick surface oxide layer.[40] This oxide layer is also thought to improve biocompatibility *in vivo* which reduces pin loosening—a factor in pin site infection.[41] The removal of this layer by polishing, as was done in some other studies, may lessen the measured difference in bacterial adhesion between the two metals.[24,35] However, there is little consensus currently on the effect of the nanostructure or the oxide layer on the biocompatibility and susceptibility to biofilm formation on titanium.[16,18,42] Although commercially pure titanium and the Ti–6Al–4V alloy used in this work have been found to absorb biomolecules differently using surface chemical techniques,[43] a recent review of the topic found no evidence that they exhibited different biocompatibilities or susceptibilities to biofilm formation.[44]

S. epidermidis and *P. mirabilis* grew biofilms on hydroxyapatite-coated K-wires to a viable cell surface density that was nonsignificantly greater than on stainless steel and 4.5× higher than on titanium alloy. Hydroxyapatite has been used as a coating on stainless steel pins to improve osseointegration and reduce pin loosening, although its effects on clinical infection rates are less clear.[26,45] The few *in vitro* studies of bacterial adhesion to and biofilm formation on hydroxyapatite coatings have produced conflicting results. Oga et al. found with scanning electron microscopy that *S. epidermidis* adhered in greater numbers to hydroxyapatite than to the uncoated metals used in this study,[46] and Ravn et al. have used microcalorimetry to reach a similar conclusion with *S. aureus*.[47] In contrast, two other microbiological studies have found that hydroxyapatite is comparatively resistant to staphylococcal adhesion.[27,48] Our finding that hydroxyapatite exhibited a similar but slightly increased propensity for biofilm formation with respect to stainless steel mirrors the similar clinical infection rate seen with hydroxyapatite and stainless steel.[45,49] The slightly greater viable cell surface density measured on hydroxyapatite in this work is likely to be because, at least in part, of its greater roughness.[47,50]

In both the microtiter plate assay and the bioreactor, *P. mirabilis* formed more biofilm than *S. epidermidis* but not significantly more ($p < 0.08$ in the crystal violet assay). This is the first time, to our knowledge, that the biofilm-forming capabilities of these two species have been compared. *P. mirabilis'* better biofilm formation may be related to its motility which allows it to swarm over implant surfaces.[51] Co-culture of *S. epidermidis* and *P. mirabilis* did not significantly increase biofilm formation beyond single culture of these species. Some other studies have shown a profound difference in biofilm formation between single and mixed species



Figs 6A to D: Biofilm growth on K-wires of different materials: (A) *S. epidermidis* in LB broth; (B) Co-culture of *S. epidermidis* and *P. mirabilis* in LB broth; (C) Co-culture of *S. epidermidis* and *P. mirabilis* in human serum; (D) Average of all results, both single culture and co-culture. The columns represent the means of the repeats (2–4 repeats in Figs A to C; 8–14 repeats in Fig. D) with error bars representing standard error. SS, stainless steel; HA, hydroxyapatite-coated stainless steel; TA, titanium alloy

cultures;[52] very weak biofilm formers can attach to, and become part of, biofilms produced by another species.[53] However, mixed biofilm interactions are species dependent and may be competitive as well as cooperative.[54] The results of this study are consistent with observations of biofilms grown on polymethyl methacrylate, indicating that any interactions between *P. mirabilis* and *S. epidermidis* lead to no significant increase in biofilm formation.[55]

The use of pure human serum as a growth medium in the bioreactor significantly increased biofilm formation compared to 10% diluted LB broth. This is probably a result of the increased nutrient levels in human serum outweighing the recently reported inhibitory effect exerted by serum proteins on biofilm growth of *S. epidermidis*.[56]

In conclusion, we compared the *in vitro* biofilm growth of clinical strains of *S. epidermidis* and *P. mirabilis* on commercially available orthopedic K-wires made from titanium alloy, uncoated stainless steel, and hydroxyapatite-coated steel. These common pin site bacterial species grew as biofilms significantly less well on titanium (as measured by the surface density of viable cells) than on the other two materials. Our results are consistent with the majority of clinical studies which have found that pin site infection rates, relative to those obtained using uncoated stainless steel, are reduced by the use of titanium and more or less unchanged by the use of hydroxyapatite-coated pins.[19–21,45,49]

Although our results are consistent with the majority of clinical studies, they are subject to the limitations of an *in vitro* study design.[57] Factors besides bacterial biofilm growth on pins were not investigated, and several such factors are doubtless involved in the development of bacterial infections at clinical pin sites (e.g., nutrient availability, immune response, and local microbiota). Mechanical factors such as wire tension and frame construction, as well as different clinical situations (e.g., nonunion or gradual deformity correction) can also be influential. *In vivo* studies are needed to measure the relative importance of these factors.

COMPLIANCE WITH ETHICAL STANDARDS

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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