Original Article

A Comparison of Bacterial Adhesion and Biofilm Formation on Commonly Used Orthopaedic Metal Implant Materials: An *In vitro* Study

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Abstract

Background: Bacterial adherence and biofilm formation on the surface of biomaterials can often lead to implant-related infections, which may vary depending on the species of microorganisms, type of biomaterial used, and physical characteristics of implant surfaces. However, there are limited studies specifically comparing biofilm formation between commonly used metallic orthopaedic implant materials and different bacterial strains. This *in vitro* study is to evaluate the ability of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* to adhere to and to form biofilms on the surface of five orthopaedic biomaterials, viz., cobalt and chromium, highly cross-linked polyethylene, stainless steel, trabecular metal, and titanium alloy. **Materials and Methods:** Bacterial adherence and bacterial biofilm-formation assays were performed by culturing *S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984, *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 for 48 h on five different biomaterials. Quantitative bacterial adherence and biofilm formation were analyzed with a scanning electron microscope. **Results:** The highest level of adherence was observed on highly cross-linked polyethylene, followed by titanium, stainless steel, and trabecular metal, with the lowest occurring on the cobalt-chromium alloy. Among the bacterial strains tested, the ability for high adherence was observed with *S. epidermidis* and *K. pneumoniae* followed by *P. aeruginosa* and *E. coli,* whereas *S. aureus* showed the least adherence. **Conclusion:** Cobalt-chromium was observed to have the lowest proclivity towards bacterial adherence compared to the other biomaterials tested. However, bacterial adhesion occurred with all the materials. Hence, it is necessary to further evaluate newer biomaterials that are resistant to bacterial adherence.

Keywords: *Bacterial adherence, biomaterials, cobalt-chromium, highly cross-linked polyethylene, stainless steel, titanium alloy, trabecular metal*

Introduction

Implant-related infections are among the most feared complications following orthopaedic procedures. The ability of the microorganisms to adhere to and to produce biofilms on the implant surface is one of the major reasons for treatment failure in implant-related infections.[1-3] Appropriate strategies aimed at preventing bacterial biofilm formation on the implant surface can aid in reducing these infections.[4-6]

The process of bacterial biofilm formation is a very complex phenomenon and is mediated by quorum-sensing molecules. The most important feature of biofilm is the production of an extracellular polymeric substance (EPS), in which bacteria are embedded and protected from various host factors. The various steps in biofilm formation include formation of a conditioning layer, adherence of bacteria, secretion of slime, and three-dimensional development, followed by maturation and detachment. Although *Staphylococcus* species, especially *S. aureus* and *S. epidermidis*, are prevalent in biofilm infections,[7,8] a variety of other pathogens including *P. aeruginosa, K. pneumoniae*, and *E. coli* can also be found.[9]

Ideal biomaterials for implants should be biocompatible with high resistance to wear, fracture, and corrosion. Only limited biomaterials standardized by the International Organization for Standardization and the American Society for Testing and Materials are available for orthopaedic implants.

Factors which influence bacterial adherence and biofilm formation on orthopaedic

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biomaterials include its chemical structure, surface roughness, Z-potential, and surface free energy.[10-18] *In vivo* proteins and host molecules of the microenvironment will be adsorbed to the biomaterial and can act as a receptor site for adhesion of tissue cells or bacteria.

Currently, a biomaterial with no bacterial adherence is unknown. Considerable efforts are being undertaken by material scientists to increase biomaterial resistance to bacterial adherence.[10] *In vitro* data on bacterial adherence and biofilm formation would help direct efforts toward the development of an infection-resistant biomaterial. This *in vitro* study involved a comparison of the degree of bacterial adherence and biofilm formation by five different common bacteria causing implant infections on five different commonly used biomaterials used to fabricate implants, thus enabling us to determine which biomaterial had minimal proclivity toward bacterial adherence and biofilm formation.

Materials and Methods

Preparation of bacteria

Standard strains of *S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603 were used as reference strains for this *in vitro* study. All strains were maintained at −80°C ± 2°C in brain–heart infusion broth (Difco Laboratories, MD, USA) supplemented with glycerol and checked for purity on sheep blood agar (BioMerieux, France) before use. Before experiments, the standard strains were subcultured into trypticase soy broth (TSB; Difco Laboratories, MD, USA) and incubated for 24 h at 37°C.

Orthopaedic biomaterial

Commercially available (Zimmer, USA) machined discs of cobalt-chromium, highly cross-linked polyethylene, stainless steel, trabecular metal (TM), and titanium with a diameter of 5 mm and thickness of 5 mm were used as substrate materials.

In vitro biofilm assays

Biofilm formation on biomaterials was assayed using the method of Braem *et al*.[19] with slight modifications. The bacteria were first inoculated overnight in 5 ml of TSB at 37°C. Overnight planktonic cultures were diluted to an optical density (600 nm, 1-cm path length) of 0.2 in TSB, giving a bacterial suspension of 1×10^4 CFU/ml. For bacterial adhesion, the biomaterial discs were then immersed in 5 ml of the bacterial suspension and statically incubated for 24 h at 37°C. Following initial bacterial adhesion, the medium was replaced with fresh TSB and further incubated for 24 h. The biofilm formed at 48 h was examined with a scanning electron microscopy (SEM).

Scanning electron microscopic study

To examine the ultrastructural nature of biofilms grown and morphological features of bacteria, sample stubs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 1 h. After washed in buffer, the samples were postfixed in 1% osmium tetroxide in the same buffer for 30 min. The samples were dehydrated in graded concentrations of ethanol and critical point-dried in CO_2 (Polaron Critical Point Dryer). They were coated with colloidal gold (Balzers SCD 050 Sputter Coater, Baltic, Liechtenstein) and viewed under a Leo 435 VP SEM (Oxford Instruments, Oxford, UK) at 15 kV.

Counting for bacterial adherence

Using secondary electron detector mode, the images of bacterial flora adhered to various biomaterials were scanned at a fixed magnification of ×5000 at an operative voltage of 20 kV and the digital images acquired. Bacterial abundance on biomaterials was counted per 2166 μm^2 area (57 μm × 38 μm) of those digital images (acquired at a fixed magnification). This counting was repeated in five replicates of the biomaterial devices that showed a consistent floral abundance and uniformly dispersed bacteria onto the surfaces. Individual bacteria were considered for counting only when their features appeared unequivocal in identification. In counting, minimal bacterial adherence was considered when bacterial abundance was <100/2166 μm^2 area and significant adherence when the abundance was 300 and above per 2166 μm^2 area examined.

Results

Using SEM, we observed that the different bacterial strains adhered at different levels on the five biomaterials. The adherence capacities of five bacterial strains to different biomaterials examined in this study are shown in Table 1.

S. aureus ATCC 29213 did not show any adherence on cobalt-chromium, titanium, and TM but was weakly adherent on stainless steel and highly cross-linked polyethylene biomaterials.

S. epidermidis ATCC 35984 showed high adherence on highly cross-linked polyethylene biomaterials [Figure 1a; average: 600/2166 μm^2 area], followed by a strong adherence on stainless steel [Figure 1b], moderate adherence on titanium, and weak adherence on cobalt-chromium and TM (50–100/2166 μm^2 area).

E. coli ATCC 25922 showed weak adherence on all five biomaterials [Figure 2].

K. pneumonia showed a moderate adherence on TM [Figure 3a], whereas weak adherence was observed on cobalt-chromium and stainless steel. A high adherence was observed on titanium [Figure 3b; 476/2166 μm^2 area] and highly cross-linked polyethylene [Figure 4; 533/2166 μm^2 area]. *P. aeruginosa* did not adhere

Table 1: Adherence capacity of *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 35984, *Escherichia coli* ATCC35218, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853 on five different biomaterials

Bacterial species	Biomaterials adherence capacity*				
	Co-Cr-Mo alloy	Titanium	Stainless steel	Trabecular metal	Highly cross-linked polyethylene
Staphylococcus aureus ATCC 29213	-	-	+	-	+
Staphylococcus epidermidis ATCC 35984	+	++	+++	+	++++
Escherichia coli ATCC 25922	+	+	+	+	+
Klebsiella pneumoniae ATCC 700603	+	++++	+	++	++++
Pseudomonas aeruginosa ATCC 27853	+	-	-	+++	+++

*-complete absence of adherence. +=Adherence<100/2166 μm^2 area, ++=Adherence of 100 and above, +++=Adherence of 200 and above, ++++=Adherence of 300 above per 2166 μm^2 area examined

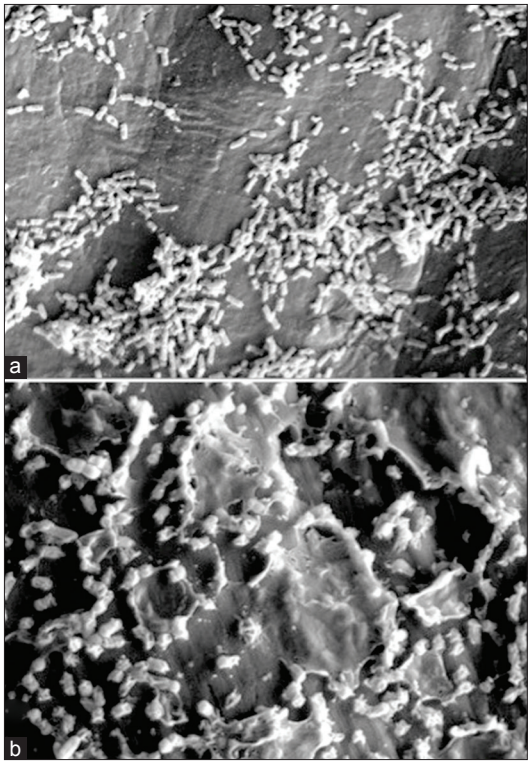

Figure 1: Scanning electron microscopic image showing high adherence by *Staphylococcus epidermidis* ATCC 35984 on highly cross-linked polyethylene biomaterial (a) and strong adherence on stainless steel (b)

on both titanium and stainless steel but adhered weakly on cobalt-chromium. However, it strongly adhered to both TM and highly cross-linked polyethylene biomaterials [Figure 5].

Discussion

Almost every pathogenic organism including bacteria, fungi, mycobacteria, and anaerobes have been shown to cause implant-associated infection.[20,21] Staphylococci have been reported as the most common pathogenic bacteria in orthopedic infections. In a large series of

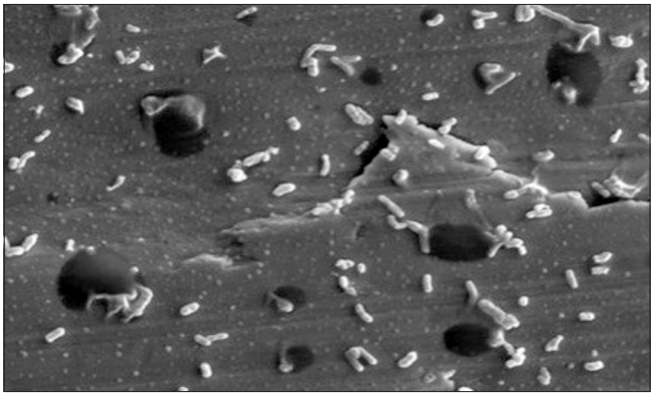

Figure 2: Scanning electron microscopic image showing weak adherence by *Escherichia coli* ATCC 25922 on cobalt-chromium-Mo alloy

about 600 prosthetic joint infections, coagulase-negative *Staphylococci*, *S. aureus*, *Streptococci,* and Gram-negative bacilli were responsible in 30%, 23%, 9%, and 6% cases, respectively. *S. aureus* sepsis has been implicated in one-third of the patients with prosthetic joint-associated infection.[21-24]

In a study by Arciola *et al*.,[7] in 2005, the authors looked at various bacteria and their prevalence in patients with orthopaedic infections with particular emphasis on implant-associated infections. They isolated 1027 microbial strains from 699 patients undergoing revision surgery. *Staphylococcus* genus was found responsible in 775 (75.5%) cases, while *Enterobacteriaceae* family, *Pseudomonas* genus, *Enterococcus* genus, and the *Streptococcus* genus were reported to be causative in 82 (8%), 75 (7.3%), 54 (5.3%), and 20 (1.9%) cases, respectively. They also reported a high prevalence of *S. epidermidis* with an incidence of 42% and 44% for infected hip and knee implants, respectively. They also reported that bacteria-infecting fracture fixation devices are different as compared to hip and knee prostheses and primarily consist of *S. aureus* and *P. aeruginosa*. *Enterobacteriaceae*, *Streptococcus*, and *Corynebacterium*

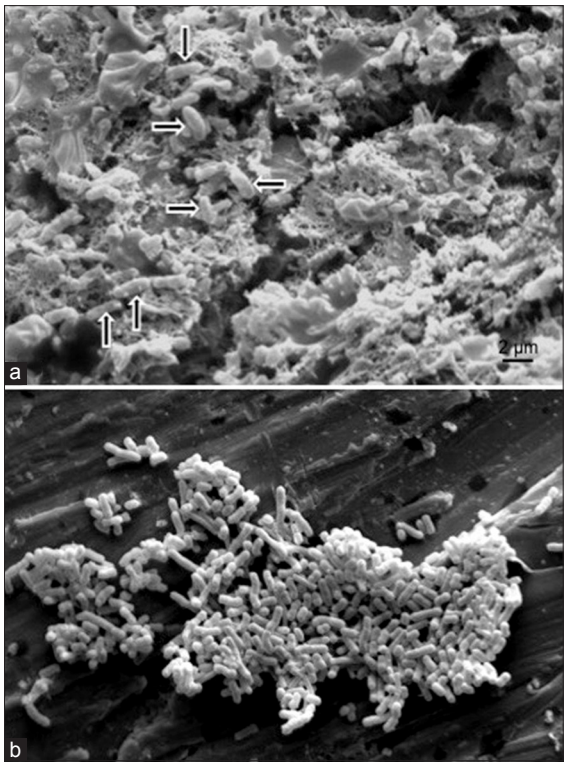

Figure 3: Scanning electron microscopic image showing moderate adherence by *Klebsiella pneumoniae* ATCC 700603 on trabecular metal (a) and high adherence on titanium biomaterial (b)

were found to be culprit in implant-associated infections where surgical incision was extending into perineal area.

Costerton *et al*.[25] wrote about two-step process of bacterial adherence and biofilm formation: first, a reversible attachment of bacteria to a biomaterial surface followed by adhesion leading to pluristratification of bacteria onto the artificial surface. In a study conducted by Wagner *et al*.,[26] they concluded that artificial surfaces are preferential adhesion sites for bacteria.

Our study tried to assess the preferential adherence of five commonly isolated bacterial strains towards five commonly used orthopedic biomaterials. Each of the biomaterials was machine cut to allow for uniform surface areas. Furthermore, all biomaterials were cultured in the different bacterial solutions for a period of 48 h, and the degree of bacterial adherence and biofilm formation was demonstrated by SEM. Various methods have been used for the detection of bacterial biofilm. However, SEM is one of the best methods to obtain information about a sample's surface topography and composition.[27] Digital image resolution of SEM is as low as 15 nm. Hence, image analysis for coating thickness and particle sizing can be done with SEM. Therefore, SEM is very useful for microstructure analysis such as evaluation of biofilm formation on orthopaedic implants.

Evaluation of the adherence property of different biomaterials showed that cobalt-chromium implants had the lowest degree of bacterial adherence and biofilm formation

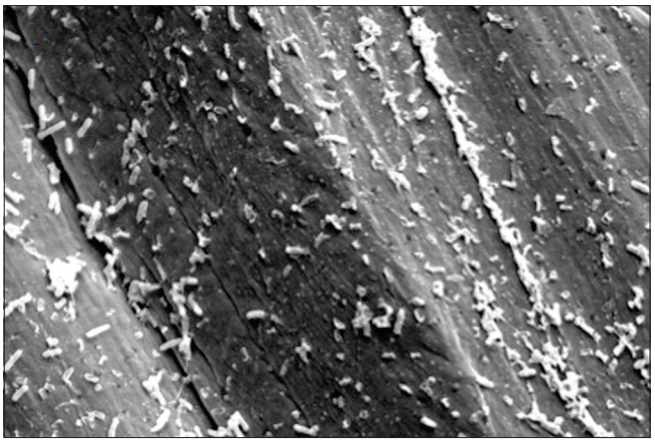

Figure 4: Scanning electron microscopic image showing high adherence by *Klebsiella pneumoniae* ATCC 700603 on highly cross-linked polyethylene biomaterial

whereas highly cross-linked polyethylene had the highest as demonstrated by SEM.

The finding of low *S. epidermidis* adhesion on cobalt-chromium alloy compared to titanium alloy and stainless steel was consistent with findings of Koseki *et al*.[28] However, our findings are in contrast to those of Patel *et al*.,[29] who observed that cobalt-chromium implants had a higher tendency for biofilm formation as compared to titanium alloy implants. Various surface properties of orthopaedic implants such as surface roughness, surface hydrophobicity, and surface free radicals, which have been extensively studied in the material science,[30,31] could have resulted in the varied outcomes observed in these studies.

Similar to the findings of Schildhauer *et al*.,[32] *S. aureus* showed no adherence to TM and weak adherence on stainless steel. However, contrary to the report of Schildhauer *et al*.,[32] who reported no significant difference in *S. epidermidis* adherence on different biomaterials, we observed that *S. epidermidis* varied in its adherence ability with maximum adherence on highly cross-linked polyethylene and weakest on both cobalt-chromium and TM. Adherence of *S. epidermidis* on stainless steel was more compared to Ti alloy as also reported by Chang and Merritt.[33]

The results of *E. coli*, *P. aeruginosa*, and *K. pneumonia* adherence on the biomaterials tested as well as those obtained from highly cross-linked polyethylene are new observations since, to the best of our knowledge, there is no published literature regarding the same. Cerca *et al*.[34] reported that adherence to biomaterials varied among strains of *S. epidermidis* and the different adherence capacity by different bacteria on the same biomaterial in our results is consistent with this observation.

This study has few limitations. The represented findings are from an *in vitro* assay and conditions may differ in the *in vivo* clinical setting. However, an insight into the ability of various bacteria for biofilm formation on

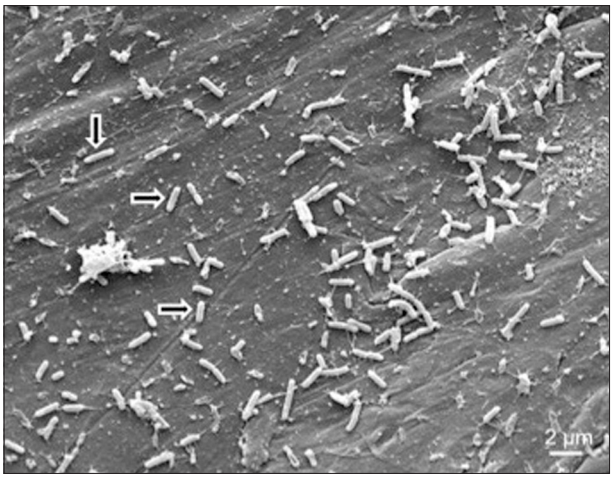

Figure 5: Scanning electron microscopic image showing strong adherence by *Pseudomonas aeruginosa* ATCC 27853 on highly cross-linked polyethylene biomaterial

different biomaterials was obtained. Although qualitative adherence was analyzed with a SEM, addition of methods such as fluorescence microscopy might have helped in quantification of bacterial adherence. Testing of both clinical and standard reference strains could have helped to achieve more realistic results. However, we believe that our study has provided valuable results in the bacterial adherence and biofilm formation on various biomaterials. The result of this study can be clinically implicated to offer benefits to choose different or alternative biomaterials in making certain clinically important decisions in many orthopaedic conditions to decrease chances of postoperative infection.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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