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RESEARCH

Evaluation of bacterial adherence of clinical isolates of *Staphylococcus sp.* using a competitive model

AN *IN VITRO* APPROACH TO THE "RACE FOR THE SURFACE" THEORY

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Objectives

Implant-related infection is one of the most devastating complications in orthopaedic surgery. Many surface and/or material modifications have been developed in order to minimise this problem; however, most of the *in vitro* studies did not evaluate bacterial adhesion in the presence of eukaryotic cells, as stated by the 'race for the surface' theory. Moreover, the adherence of numerous clinical strains with different initial concentrations has not been studied.

Methods

We describe a method for the study of bacterial adherence in the presence of preosteoblastic cells. For this purpose we mixed different concentrations of bacterial cells from collection and clinical strains of staphylococci isolated from implant-related infections with preosteoblastic cells, and analysed the minimal concentration of bacteria able to colonise the surface of the material with image analysis.

Results

Our results show that clinical strains adhere to the material surface at lower concentrations than collection strains. A destructive effect of bacteria on preosteoblastic cells was also detected, especially with higher concentrations of bacteria.

Conclusions

The method described herein can be used to evaluate the effect of surface modifications on bacterial adherence more accurately than conventional monoculture studies. Clinical strains behave differently than collection strains with respect to bacterial adherence.

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Keywords: Bacterial adherence, Preosteoblastic cells, Implant-related infection

Article focus

- *In vitro* bacterial adherence studies are usually performed only with bacterial cells. A combination of bacterial and eukaryotic cells is a more realistic approach for this purpose. No broad study that evaluates different types of bacterial strains in co-culture with eukaryotic cells has been performed to date.

Key messages

- We present an easy and reproducible co-culture method to evaluate the 'race for the surface'.
- Clinical strains show different behavior than collection strains, being more adherent in the *in vitro* model.
- Clinical strains also showed differences between them, which suggests the need for evaluation of several strains in the *in vitro* adherence studies in order to have more realistic results.

Strengths and limitations

- The study is easy to perform, reproducible and can be used with different types of bacteria.
- The main limitation of this study is that the methodology cannot be considered identical to pathogenic processes of implant-related infection. For instance, the addition of macrophages, which play a key role in phagocytosis, should be take into account for the development of further studies.

Introduction

According to the 'race for the surface' theory, the presence of a foreign body triggers a race between tissue and bacterial cells for the colonisation of the implant. If tissue cells win this race, the latter surface is less vulnerable to bacterial colonisation. On the other hand, if bacteria win the competition, the implant surface will eventually become covered by a biofilm and tissue cell functions will be impaired by bacterial toxins.[1-4] Given the resistance of biofilms to host defenses and conventional antimicrobial agents, most implant-associated infections (IAI) have a chronic course and are responsible for implant failure. Removal of the prosthesis, followed by debridement and re-implantation, is frequently the only therapeutic option.[5-8] Therefore, the search for biomaterials and strategies that allow proper tissue integration and prevent bacterial adhesion is an issue of great concern.

Bacterial adhesion and biofilm formation on implants and coatings have been traditionally studied separately from tissue cell adhesion and integration, i.e., biomaterials or coatings are usually either evaluated for their ability to resist bacterial adhesion or to support tissue cell adhesion and integration.[2,9-11] Hence, the combined outcome of these two interactions, the 'race for the surface', remains poorly known.[12] *In vitro* co-culture studies could provide more realistic conclusions than those obtained from these monoculture studies with either bacteria or tissue cells.[13,14]

It has been reported that the outcome of this race seems to be dependent on bacterial strain, on the amount of bacteria present on the implant prior to cell seeding,[12] and on the local immune response and the properties of the implant surface.[15,16]

In this work, we performed a new experimental co-culture system to evaluate the impact of eukaryotic cells on the pathogenesis of implant-related infections. It is a simple and reproducible method to study both bacterial and osteoblast adhesion to the biomaterial simultaneously. We also assessed the influence of several laboratory and clinical strains of staphylococci on the 'race for the surface'.

Materials and Methods

Bacterial growth. Bacterial studies were performed with *Staphylococcus epidermidis* and *Staphylococcus aureus*. Two laboratory *(S. epidermidis* American Type Culture collection (ATCC, Manassas, Virginia) 35984 and *S. aureus* 15981 (17)) and six clinical (*S. aureus* P1, P2 and P18; *S. epidermidis* P33, P55 and P101) strains were used.

Clinical strains were isolated by a sonication procedure[18] from hip prostheses (P2, P18, P33, P55 and P101) and osteosynthesis implants (P1). Briefly, samples inside rigid plastic bag containers were sonicated with Ultrasons-H 3000840 low-power (50/60 Hz, 200 W) bath sonicator (J. P. Selecta, Abrera, Spain) for five minutes; then, the sonicate was centrifuged at 3000 x *g* for 20 minutes and the sediment was re-suspended in sterile phosphate buffered saline (PBS) and inoculated onto Tryptic soy- 5% sheep blood agar, chocolate agar, MacConkey agar, Schaedler agar, Middlebrook7H10 agar plates and Sabouraud-chloramphenicol agar tubes (BioMérieux S.A., Marcy l'Etoile, France). Although contaminations have been described in sonication with plastic bags,[19] no leaks or ruptures were detected in a detailed exam before and after the procedure. Moreover, other measures to reduce the possibility of contamination were taken, such as the use of newly added sterile distilled water for each sonication and emptying the sonicator between uses to avoid bacterial overgrowth in the water. The five minute sonication time was chosen as Kobayashi et al[20] recommend that a sonication time of one to five minutes is ideal for dislodging biofilm bacteria without affecting bacterial viability. We have selected these strains because they were considered the actual cause of the prosthetic joint infections.

Isolated organisms were identified according to commonly used commercial biochemical tests (API-Staph strips; BioMérieux S.A.) and MALDI-TOF technology (Vitek MS; BioMérieux S.A.). A genetic study[21] showed that all strains except P33 and P101 expressed intercellular adhesion, icaA and icaD, genes. Strains P1 and P55 also expressed the Intercellular Adhesin Locus Regulator (icaR) gene. Strain P33 is a heavy biofilm producer, while all the other strains were low biofilm producers as determined by the Stepanovic test.[21]

For each experiment, strains were streaked on a blood agar plate from frozen stocks and grown overnight at 37°C in a humidified 5% CO_2 atmosphere. Two colonies were then inoculated in 9 mL of Trypticase Soy Broth (TSB-T; BioMérieux S.A.) and cultured for 24 hours in the above-mentioned conditions (5% CO_2, 37°C). Subsequently, bacteria were harvested by centrifugation at 3000 x *g* for 10 minutes at room temperature and washed three times with 1 mL of phosphate-buffered saline (PBS) by centrifugation.The final pellet was resuspended in 1 mL of sterile PBS and diluted with PBS to a concentration of 10^8 colony-forming unit (CFU)/mL. Finally, starting from the previous suspension, five 1:10 serial dilutions were prepared in PBS (10^8, 10^7, 10^6, 10^5, 10^4 and 10^3) and CFU/mL.

Preosteoblastic cell culture. Cell culture experiments were performed using the well-characterised mouse preosteoblastic cell line MC3T3-E1 (subclone 4, CRL-2593; ATCC, Mannassas, Virginia). These cells have shown to differentiate into mature osteoblasts as occurs during *in vivo* bone formation.[22] Cells were routinely cultured in α-Minimum Essential Medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher scientific, Waltham, Massachusettes), 1% penicillin and streptomycin, and incubated at 37°C in a humidified 5% CO_2 atmosphere. When cultures were

90% to 100% confluent, cells were detached by using 0.5% trypsin-0.2% EDTA and harvested by two minutes centrifugation at 3000 rpm.

Viable cells (assessed by trypan blue exclusion) were counted in an automatic-cell counter (Countess, Thermo Fisher Scientific). In order to avoid genetic drifts, no cell passages (subcultures) over number 15 were used for the experiments described below.

For co-culture experiments, a suitable medium for optimal growth of both preosteoblastic cells and bacteria was selected. Initially, subconfluent MC3T3-E1 cells (10^5 cells/cm^2) were incubated in α-MEM and 1% FBS, diluted or not with TSB-T medium at 25%, 50% or 75% for 24 hours. TSB-T was selected because it is a good culture medium for bacterial strains and was used after an evaluation regarding its null effect on the eukaryotic cells. Growth rates of both bacteria and preosteoblastic cells were also determined with white light microscopy using different concentrations of cells. Cell death, assessed by trypan blue staining, was significant in the presence of 75% TSB-T medium. The medium composition showing optimal *Staphylococcus* s, pp and MC3T3-E1 cell growth, 50% TSB-T, was chosen for further studies.

Preosteoblasts-bacteria competitive assay. An 18-mm diameter rod of Ti–6Al–4V alloy ELI grade according to the standard ASTM F136-02 supplied by Surgival Trauma Sociedad Limitada (Vila-Seca, Tarragona, Spain) was cut into 2-mm thick disk specimens, ground through successive abrasive grinding papers of Silicon Carbide (SiC) from 60 to 1200 Standard ANSI grit, degreased with a conventional detergent, and rinsed in tap water followed by deionised water (Mean roughness was 178.94 nm). The specimens were subsequently chemically polished (CP) on one side (treated area: 2.54 cm^2) in a mixture of 48% hydrogen fluoride (HF): 70% nitric acid (HNO3):water (1:4:5 v/v) for five minutes at room temperature under continuous agitation at 400 rpm, rinsed in distilled water and dried in cold air.[23]

Nine CP disks were placed into Petri dishes and covered with different solutions: six of them were covered with 2 mL of one of the aforementioned bacterial dilutions and 2 mL of MC3T3-E1 cells at a fixed concentration of 10^5 cells/mL. We have selected this concentration in previous tests as the most ideal concentration to perform a proper interpretation of the images. The three remaining CP disks were used as controls: a bacterial control composed of 4 mL of the 10^8 CFU/mL bacterial solution, a preosteoblastic cell control with 4 mL of MC3T3-E1 cells at 10^5 cells/mL and a negative control with 4 mL of PBS. After incubation for six hours at 37°C in a humidified 5% CO2 atmosphere under static conditions, the disks were washed three times with PBS to remove the unbound cells, as follows: for each disk, three Petri dishes with 10 mL of sterile PBS were placed, and disks were shaken with sterile tweezers in each of them. All manipulations were performed by the same person (MMP), aiming to reduce the variability. Subsequently, the disks were stained with acridine orange (AO) (BD, Franklin Lakes, New Jersey) for two minutes, and then rinsed with sterile water (to get rid of the excess of dye). AO stained bacterial cells in a bright orange, while eukaryotic cells acquired a subtle greenish-orange colour. By using a Leitz LaborLux D fluorescence microscope (Leica Microsystems Inc, Buffalo Grove, Illinois), 16 images of the surface of each disk were randomly taken with a digital camera Nikon CoolPix 8400 (Nikon Corporation, Minato, Tokyo, Japan): eight of the images focused on preosteoblastic cells at 200 x magnification and the remaining for assessing bacterial adherence, at 400 x magnification. Cells were counted per field. Partially visible cells were taken into account if at least half of the nucleus appeared on the picture.

The percentage of bacterial surface coverage was analysed with the picture-processing Image J software (National Institutes of Health, Bethesda, Maryland), which counted the gross fluorescent surface area corresponding to the bacteria. Experiments were performed in triplicate for each strain and dilution.

Statistical analysis. Statistical analysis was performed with EPI INFO 7.0 software (Centers for Disease Control and Prevention, Atlanta, Georgia). Bartlett's Test was used for the evaluation of the inequality of population variances. A Mann-Whitney U test was applied for comparisons between two groups, whereas for more than two groups Kruskal-Wallis test was chosen. Moreover, Bonferroni correction was used for *post hoc* comparisons between groups.

Results

Co-culture experiments

Bacterial adhesion of collection strains. Bacterial adhesion to CP disks was observed for the experiments performed with 10^6, 10^7 and 10^8 CFU/mL for both *S. aureus* 15981 and *S. epidermidis* ATCC 35984 collection strains (Fig. 1). Bacterial concentrations below 10^6 CFU/mL showed no detectable bacterial adhesion to this titanium alloy. *S. epidermidis* showed better adhesion to CP disks than *S. aureus*, and this higher percentage of covered surface by the former strain was statistically significant with 10^8 CFU/mL ($p < 0.0001$, Kruskal-Wallis test).

Bacterial adhesion of clinical strains. Bacterial adhesion to CP disks was observed for all dilutions (10^8-10^3 CFU/mL) with all the *S. aureus* clinical strains evaluated (Fig. 1): P1, P2 and P18. However, *S. aureus* showed great variability in adherence to CP disks between the three clinical strains tested; differences were statistically significant when comparing 10^7, 10^6, 10^4 and 10^3 CFU/mL ($p < 0.016$, Kruskal-Wallis test). Thus, while P2 showed maximal adhesion at 10^8 CFU/mL, P1 and P18 presented

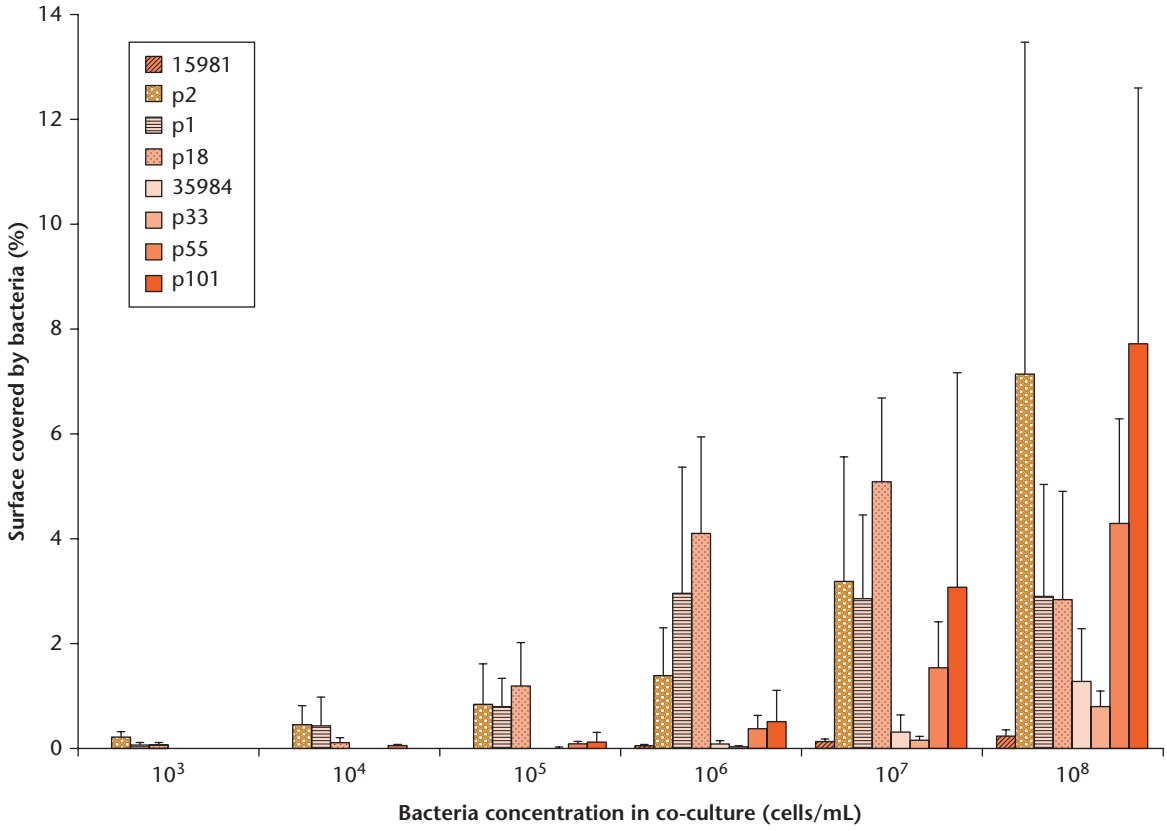

Fig. 1

Bacterial adherence of all collection and clinical strains tested. This figure represents the percentage of surface covered by the tested bacteria that adhered to the chemically polished (CP) disks in the co-culture experiments. Osteoblast concentration used was the same for each experiment (10^5 cells/mL).

maximal adhesion to CP disks at 10^6 and 10^7 CFU/mL, respectively.

The minimal concentration of *S. epidermidis* needed to adhere to CP disks varied with the strain (Fig. 1): P33 and P101 showed no detectable adhesion to CP disks below 10^5 CFU/mL, whereas no bacterial adhesion was observed below 10^4 CFU/mL for P55. *S. epidermidis* showed the highest percentage of area covered for P101, followed by P55 and P33, which presented the lowest adhesion. These differences in adhesion were statistically significant when making a global comparison of the strains. By comparing them separately (two to two), we found that the differences were also significant, except for P55 and P101 in the cases of 10^3 to 10^7CFU/mL.

Adhesion of MC3T3-E1 cells in the presence of bacterial collection strains. Preosteoblastic MC3T3-E1 cell adhesion to CP disks was observed for all solutions tested, with or without bacteria (Fig. 2). Although cell attachment was significantly different for several combinations with different bacterial concentrations, they did not follow a consistent pattern.

The number of adhered MC3T3-E1 cells was maximal at 10^6 CFU/mL for both bacterial species. Surface attachment was higher ($p < 0.05$, Mann-Whitney U test) for preosteoblastic cells in the presence of *S. epidermidis* 35984 than with *S. aureus* 15981 at 10^7, 10^6 and 10^5 CFU/mL.

Adhesion of MC3T3 cells in the presence of bacterial clinical strains. Preosteoblastic cells adhesion to the CP disks was also observed for solutions made with all bacterial dilutions (Figs 2 and 3). Although the number of cells tended to vary among strains, these differences were not significant. We observed that cell adhesion on CP disks was random.

Discussion

The success of prosthetic surgery is based not only on the absence of bacterial adhesion and biofilm formation, but also on the correct tissue integration.[1] Considering that some orthopaedic implants could be inserted in a bacterially contaminated tissue (especially osteosynthesis implants),[24] host tissue cells have to compete with bacteria for correct implant integration.[25] A plausible *in vitro* approach to this problem pertaining to prosthetic devices may involve the use of bone cells in combination with bacteria to assess bacterial adhesion to biomaterials. However, these co-culture studies have seldom been reported. In this regard, the race for the surface model previously established by Gristina[1] has conceptualised the fate of an available (implant) surface as a contest between tissue cell integration and bacterial adhesion to that same surface.

The practical impact of such a concept comes from the consideration that a number of biomaterial models in

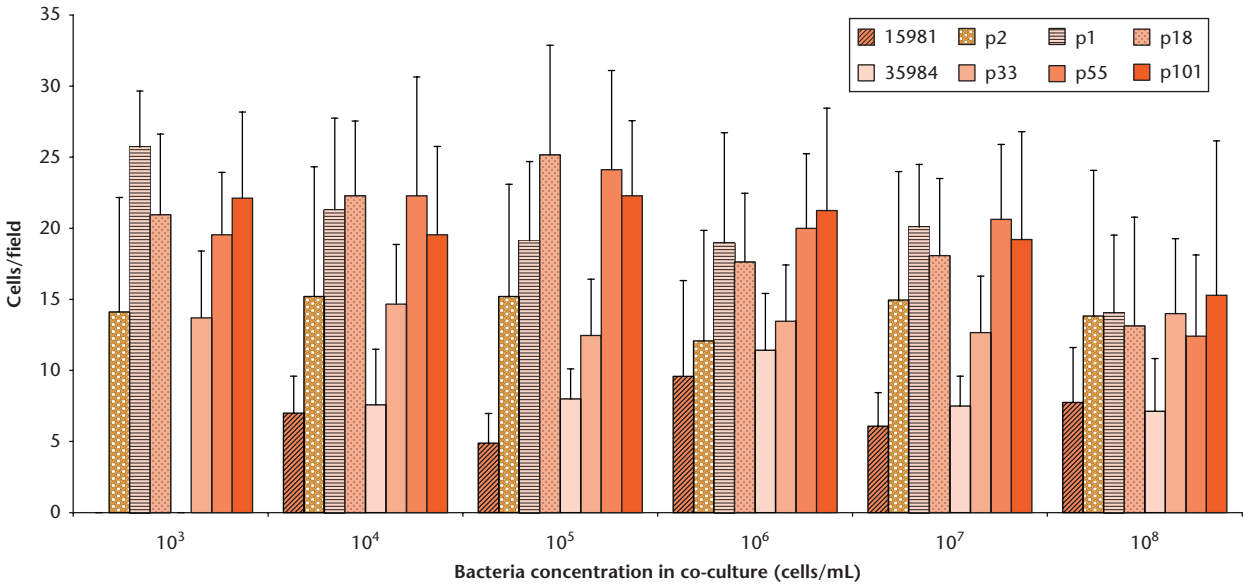

Fig. 2

Pre-osteoblastic cell adherence in co-culture with collection and clinical strains.

development for reducing bacterial adherence could exhibit poor tissue integration behavior. Thus, the main objective of the present study was to develop a cell model as a suitable *in vitro* surrogate for the race for the surface between bacteria and host tissue cells using a simple experimental setting. Trying to approach biomaterial-related infection in a more realistic way than conventional adherence studies, we have developed a co-culture system that takes into account the role of both tissue cells and commonly infectious bacteria in IAI. For the development of the optimal growth medium to perform the experiments, Subbiahdoss et al[12] considered the changes in morphology (and subsequent death) of the U2OS, human osteosarcoma cells, as the most important factor. Thus, they used a modified culture media in which these cells did not undergo changes in morphology and had a growth advantage over bacteria. In contrast, we selected experimental culture conditions that prevented any advantage for bacterial *versus* preosteoblastic cell growth. Moreover, we wanted to evaluate bacteria and MC3T3-E1 cell adhesion to the biomaterial when reaching the surface simultaneously. This was unlike other previously reported studies, in which the material was infected prior to the adhesion of eukaryotic cells[12,13,26-29] (so that bacteria had an advantage to win the race from the beginning), or after the integration of the tissue on the implant (so that cells had such an advantage).[30]

Furthermore, this model was not designed to require a flow chamber device, which require setting up and availability, so it provides both simplicity and reproducibility. However, for some studies with long duration, the flux of blood and other fluids in the prosthesis must be taken into account. We studied a six-hour incubation in co-culture, time enough to evaluate the adhesion of both preosteoblastic and bacterial cells present in the first steps of to the surface. We could, therefore, analyse the behavior of diverse strains and the effect of different initial concentrations (from 10^8 to 10^3 CFU/mL). However, it would also be of interest to conduct the same study with a longer time of incubation, as has been performed in other studies.[12,13,31]

An important aspect in wound healing and implant integration is that, upon implantation, the surface of the prosthetic device is in contact with the blood of the patient. Proteins and blood components create an extracellular matrix known to be of high importance in cell adhesion and activation of ligands.[32,33] For the improvement of further experiments, and achieving an approach that is closer to real physiological conditions, the addition of whole blood or serum to our experiment could be of high interest.

Our study was carried out with both clinical and laboratory *S. aureus* and *S. epidermidis* strains, which both account for more than half of the IAI in early and late infections during total knee and hip arthroplasty.[7,34-36] This fact can be supported by the ability of these species to adhere to biomaterial devices and to form a biofilm.[37,38] Most studies on bacterial adhesion to biomaterials have been performed using only collection strains, which are adapted to laboratory conditions and may lack the pathogenic features currently present in wild-type strains.[39] Clinical strains, however, possess a different genetic load and pathogenic factors, so their behavior might be different to that of laboratory-adapted strains.[40,41] This study demonstrates the different adherence abilities of these strains compared with the collection strains. Whilst the latter needed a higher inoculum (at least 10^6 CFU/mL) in order to adhere to the titanium alloy in the presence of

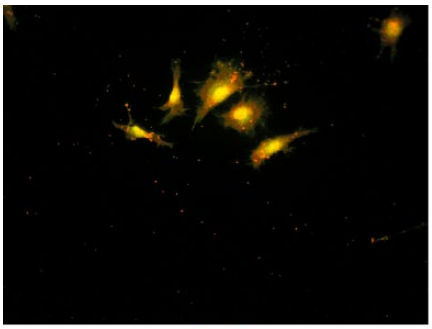
Fig. 3a

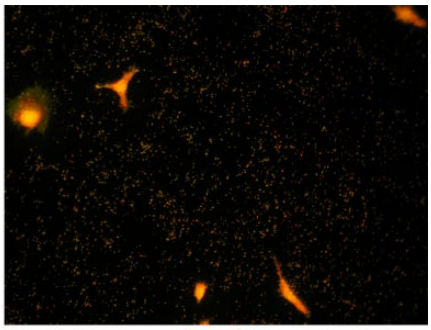
Fig. 3b

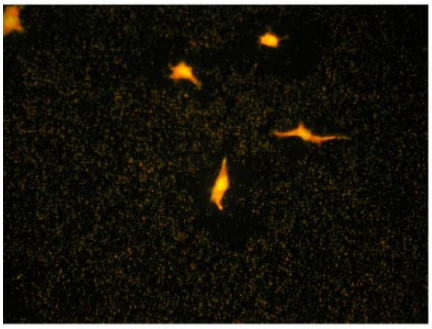
Fig. 3c

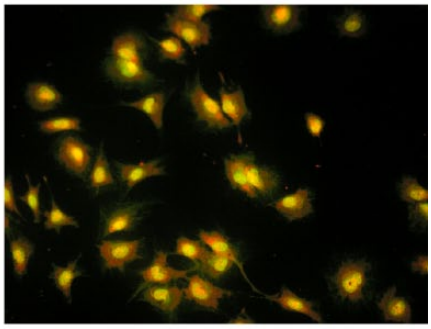
Fig. 3d

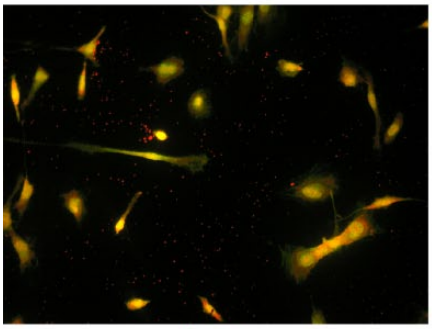
Fig. 3e

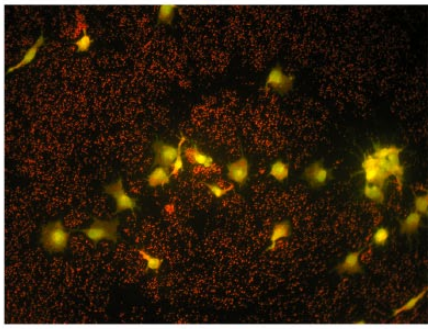
Fig. 3f

Representative fluorescence microscope images of adherence of clinical strains in the presence of pre-osteoblasts. 3A-C: *S. aureus* strain P2 (concentrations: A-10^4, B-10^6, C-10^8). 3D-F: *S. epidermidis* strain P55 (concentrations: D-10^4, E-10^6, F-10^8). 200x magnification.

osteoblasts, clinical strains adhered with lower concentrations. Furthermore, the adhesion of the two collection strains tested was very low even for the highest concentration; this percentage was considerably higher for any of the clinical strains. These results emphasise that the study of clinical strains is essential for a valuable evaluation of biomaterials.

Because the actual bacterial load that causes infection is still unknown, previous studies to assess bacteria colonisation of the implant surface have used bacterial concentrations in the range of 10^3 to 10^6 CFU/mL.[12,13,25-28,12,13,26-29] In this study, we chose five concentrations for collection strains (10^4, 10^5, 10^6, 10^7, 10^8 CFU/mL) but six (10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/mL) for clinical strains as they show high adherence to the surface, in the presence of a constant concentration of osteoblasts (10^5cells/mL). Although most of the bacteria strains tested had maximal adhesion to CP disks at 10^8 CFU/mL, for P1 and P18 this corresponding value was attained at 10^6 and 10^7 CFU/mL, respectively. Such a high order of magnitude in different concentrations was expected to correspond to differences in the percentage of material surface covered with each bacteria strain. In this scenario, for some strains (P1 and P18), preosteoblastic cells might have taken advantage in the race for the surface, thus displacing bacteria that otherwise (without osteoblasts) might have been able to adhere to the material surface. This explanation can also be applied to P33 *S. epidermidis* strain.

Since we wanted to evaluate the impact of the initial concentration of bacteria, we chose a constant preosteoblastic cell concentration. The results showed that adhesion of preosteoblasts to the disks did not follow any pattern. Although these cells showed their maximal adhesion at 10^6 in co-culture with collection strains, we think that this could be the actual concentration when osteoblasts could start to win the race against bacteria, but we didn't observe this phenomenon in the case of

clinical strains. Therefore, we attributed this fact to an occurrence of chance.

Other studies can be performed with this model, such as intracellular invasion of osteoblasts, a fact that could have extreme importance in the management of chronic infections,[42] as well as studies of surface modifications in order to minimise bacterial colonisation of the biomaterial. Moreover, similar models have also demonstrated the usefulness of this approach for a proper *in vitro* study of bacterial interactions during the process of biomaterial related infection.[43]

In conclusion, this study represents a new *in vitro* approach to the 'race for the surface' theory. The development of new biomaterials or functional coatings that resist bacterial adhesion and support tissue cell adherence at the same time requires a deeper knowledge of eukaryotic-bacteria cells interactions. For this purpose, we developed a simple and reproducible *in vitro* model that evaluates simultaneous tissue cell adhesion and spreading and bacterial growth on a commonly used material for prosthetic devices. Applying this experimental maneuver to different laboratory and clinical bacterial strains, the results show a great variability between them, with respect to both the concentration needed to adhere to the titanium alloy surface (effect of initial concentration) and the amount of bacteria adhered (initial outcome of the race for the surface). This method might be useful for assessing the impact of surface modifications to obtain new materials with antibacterial properties.

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Author Contribution

- M. Martinez-Perez: Optimised the model, performed the microbiological studies and image analysis, participated in the manuscript design and writing and approved the final version.
- C. Perez-Jorge: Optimised the model, performed the microbiological studies and image analysis, participated in the manuscript design and writing and approved the final version.
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- S. Portal-Nuñez: Optimised the model and performed the cell cultures and studies with eukaryotic cells, participated in the manuscript design and writing and approved the final version.
- R. Perez-Tanoira: Optimised the model, performed the microbiological studies and image analysis, participated in the manuscript design and writing and approved the final version.
- A. Conde: Developed and provided the material and the analysis of the different material samples. Manuscript preparation.
- M. A. Arenas: Supervised the study and collaborated with ideas for the design of the study, participated in the manuscript design and writing and approved the final version.
- J. M. Hernandez-Lopez: Developed and provided the material and the analysis of the different material samples, participated in the manuscript design and writing and approved the final version.
- J. J. de Damborenea: Developed and provided the material and the analysis of the different material samples. Manuscript preparation.
- P. Esbrit: Fabricated and provided the material and the analysis of the different material samples, participated in the manuscript design and writing and approved the final version.
- J. Esteban: Had the idea of the study, participated in the design, and supervised all the *in vitro* study and the analysis of the data, participated in the manuscript design and writing and approved the final version.

ICMJE Conflicts of Interest

- None declared