In vivo monitoring of *Staphylococcus aureus* biofilm infections and antimicrobial therapy efficacy by $^{18}$F-FDG-MicroPET in a mouse model

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Running head: *S. aureus* biofilm monitoring by FDG-MicroPET in mice

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Abstract

A mouse model was developed for in vivo monitoring of infection and antimicrobial treatment efficacy against Staphylococcus aureus biofilms, using $^{18}$F-FDG-MicroPET image technique. In the model, sealed Vialon™ catheters were shortly pre-colonized with *S. aureus* strains ATCC 15981 or V329 that differ in cytotoxic properties and biofilm matrix composition. After subcutaneous implantation of catheters in mice, the *S. aureus* strain differences found in bacterial counts and inflammatory reaction triggered were detected by the regular bacteriological and histological procedures and also by $^{18}$F-FDG-MicroPET image signal intensity determinations, in the infection area and regional lymph node. Moreover, $^{18}$F-FDG-MicroPET image allowed the monitoring of the rifampin treatment efficacy, identifying the periods of controlled infection and those of reactivated infection due to the appearance of bacteria naturally resistant to rifampin. Overall, the mouse model developed may be useful for non-invasive in vivo determinations in studies on *S. aureus* biofilm infections and assessment of new therapeutic approaches.

Key words: *Staphylococcus aureus*, biofilm, catheter, mice, MicroPET, $^{18}$F-FDG
Introduction

Often, *in vivo* bacterial biofilms are inadvertently undetected since viable bacteria are only shed periodically. This hampers diagnosis, which often requires puncture biopsy and/or surgery with the associated cost, morbidity and mortality. *Staphylococcus aureus* is one of the most frequent device associated pathogens. In staphylococcal biofilms, bacterial cells are interconnected by different types of polymeric matrices; among others, those composed by PIA/PNAG [Polymeric N-acetyl-β-(1,6)-glucosamine] or proteins such as Bap (1-7). Of these, PIA/PNAG matrices are particularly common in clinical isolates (2) and medical device-related biofilm infections by *S. aureus* (mainly MSSA) and *Staphylococcus epidermidis* (8, 9).

Subcutaneous catheters have been successfully used in models that simulate natural device-related staphylococcal infections. However, with the use of non-sealed catheters in these models, the biofilm may be developed inside the catheter lumen, allowing only a partial interaction between bacteria and the immune system cells (10, 11).

Although *S. aureus* biofilm infections have been widely characterized and quantified, especially *in vitro*, an experimental non-invasive model to assess biofilm infections by natural or genetically modified bacteria is still needed to monitor *in vivo* infection and treatment efficacy in preclinical studies. One of the most useful animal models developed so far requires the use of bioluminescent *S. aureus* bacteria genetically modified to contain *lux* genes (12, 13). The use of this method is particularly limited when infections are caused by non-bioluminiscent bacteria, or in long-term studies where loss of bioluminescence may occur (14).

Positron emission tomography (PET) with 18F-Fluoro-DeoxyGlucose (18F-FDG), an *in vivo* nuclear-medicine imaging technique requiring the administration of this glucose analog
radiotracer, is being successfully applied in clinical infections, since this radiotracer is metabolized by many infectious and inflammatory cells (15). This technique has been proposed for early detection of different infectious processes (16) and also in combination with magnetic resonance imaging for clinical and biomedical research (17). The main advantages of PET over other imaging techniques are its high sensitivity and the possibility to perform quantitative measurements in the images generated. In animals, $^{18}$F-FDG-MicroPET has been used in different rabbit osteomyelitis models (18, 19). The most recent one was performed to detect staphylococcal infections in bone and compare *S. aureus* vs. *S. epidermidis* infections (18).

The aim of our work was to design a simple protocol in mice that would allow the *in vivo* non-invasive $^{18}$F-FDG-MicroPET imaging technique for assessment of *S. aureus* strain differences and monitoring of either infections in untreated animals or the antimicrobial treatment efficacy in longitudinal studies.

**Material and methods**

**Bacterial strains and culture conditions**

Two *S. aureus* wild-type strains (named ATCC 15981 and V329) having a high capacity to form biofilms but triggering different types of infections (clinical and subclinical, respectively) were used. Strain ATCC 15981 was from a human clinical otitis patient. This *bap*-negative strain contained genes encoding exocellular proteases (20) and formed a highly adherent hyperbiofilm with an *ica*-dependent PIA/PNAG polysaccharidic matrix (21). Strain V329 was from bovine subclinical mastitis (1), produced a Bap-protein biofilm matrix (21) expressed in a stable *ica*-independent manner *in vivo* (22) and had a very low pathogenicity in experimental animal infections (23). These strains were kindly provided by
Drs. I. Lasa (Instituto de Agrobiotecnología, Pamplona, Spain) and J. Penadés (University of Glasgow, Glasgow, UK), respectively.

*S. aureus* bacteria were cultured (37°C, 18 h) in Tryptone Soy Agar (TSA; Laboratorios Conda, Spain) or Tryptone Soy Broth (TSB; Laboratorios Conda, Spain) supplemented with glucose 0.25% (TSA-glc and TSB-glc). Bacterial concentration was first adjusted, spectrophotometrically and by dilution in TSB-glc, to $1 \times 10^5$ bacteria/mL for catheter infection. Exact doses (CFU) were retrospectively assessed by serial ten-fold dilutions in Phosphate Buffer Saline (PBS; pH 7.4) and plating 100 µL by triplicate in TSA-glc (37°C, 18 h).

**Mouse model of *S. aureus* sealed catheter infection**

Female CD1 mice (Charles River International) of 20-22 g body-weight were accommodated in the animal facilities of the Universidad Pública de Navarra (UPNA; registration code ES/31-2016-000002-CR-SU-US), with water and food *ad libitum*. Mice handling and procedures were performed in compliance with the current European and national regulations, following the FELASA and ARRIVE welfare guidelines, and with the supervision of the UPNA’s Comité de Ética, Experimentación Animal y Bioseguridad (CEEAB) and approval by the competent authority (Gobierno de Navarra).

To prepare implants, commercial Vialon™ 18G 1.3 × 30 mm catheters (Becton-Dickinson) were cut into 20 mm segments, and sealed with under sterile conditions with vaseline and tissular glue (Vetbond®, 3M España S.A.). Cleaning and disinfection were achieved thereafter by immersion in DD445 (A&B Laboratorios de Biotecnología) and ethanol (15 min in each solvent). Sterility was checked by incubation (37°C, 24 h) in TSB. Then, reliable *S. aureus* catheter pre-colonization was successfully achieved by incubation (37°C, 4 h) in 1 mL TSB-glc containing $1 \times 10^5$ CFU, as previously reported (10). The number of
bacteria adhered to implants prior to infection was systematically assessed. Finally, catheters were rinsed with fresh TSB-glc and immediately implanted subcutaneously through a minimal surgical incision in the interscapular area of mice, previously anaesthetized by intraperitoneal administration of ketamine (100 mg Kg\(^{-1}\); Imalgene\(^{\circledR}\), Merial Laboratorios, S.A.) and xylacine (10 mg Kg\(^{-1}\); Rompun\(^{\circledR}\), Bayer Health Care). In each experiment, non-precolonized but TSB-glc treated catheters were subcutaneously implanted in control mice.

**Experimental design**

Following implantation, four main animal experiments were carried out. Detailed description of each experiment is included in sections below and animal group distribution and assessment time-points are summarized in Fig. 1.

**Clinical and bacteriological studies**

After catheter implantation in groups of 50 CD1 mice, animals were examined daily by palpation for the presence of catheter and signs of local infection (Fig. 1A). At days 1, 2, 3, 4, 7 and then weekly for 6 weeks post-implantation (PI), 5 mice per bacterial strain were euthanized by cervical dislocation and catheters were aseptically removed, homogenized in 1 mL PBS by ultrasound bath (40-50 Hz, 30 min; Selecta) and vortexed (1 min). The number of CFU adhered to the catheter was determined by serial ten-fold dilution of homogenates in PBS, plating 100 µL by triplicate in TSA, and incubation of plates (37°C, 18-24 h). Finally, the mean and SD (n=5) of log\(_{10}\) CFU/catheter was calculated.

**Histopathological analysis and lesion score**

Sealed catheters pre-colonized with *S. aureus* strains ATCC 15981 or V329, as described above, were implanted in groups of 15 CD1 mice per strain (Fig. 1B). Additional mice (n=15) carrying sealed catheters pre-incubated in TSB-glc alone were used as uninfected
controls. Tissue samples containing the catheter and surrounding tissues (adipose, muscular, connective) were obtained at 1, 7 and 14 days PI and fixed for 24 h in 10% buffered formaldehyde solution. Five transaxial slices obtained every 2-3 mm were embedded in paraffin. Then, 4-6 μm sections were stained with haematoxylin and eosin (HE) by standard procedures and examined for four histological features: (1) local inflammatory reaction by infiltration of polymorphonuclear leukocytes or mononuclear cells around the catheter; (2) cellular debris and acellular exudates between the catheter and the inflammatory reaction; (3) fibrosis and encapsulation of the reaction around the catheter; and (4) diffuse inflammatory reaction located between the adipose, muscular and other peripheral tissues. Lesions were scored on a scale of 0 to 4 (0: absent to very low; 1: mild; 2: moderate; 3: strong; 4: very strong). Similar sections were stained for immunohistochemical (IHC) studies to specifically detect the antigens PIA/PNAG (strain ATCC 15981) or Bap (strain V329). These sections were subjected to antigen retrieval by heating the slides in a pressure cooker for 3 min in distilled water, pH 4.0. The IHC staining was performed using polyclonal rabbit antibodies to PIA/PNAG and Bap, kindly provided by Drs. J. Pier (Harvard School, USA) and J. Valle (CSIC-Instituto de Agrobiotecnología, Spain), respectively. Primary antibodies were diluted 1:750 (PIA/PNAG) and 1:500 (Bap) with Dako Antibody Diluent (Dako Denmark A/S), and the reaction detected by using a DAKO HRP rabbit secondary antibody (Dako, Denmark A/S). Tissue preparations from mice carrying a non-infected sealed catheter were used as control. Additionally, some tissue sections obtained from mice infected (see above) with the corresponding S. aureus biofilm were incubated with conventional rabbit serum as primary antibody (additional control). Images were observed and digitalized using an Olympus Vanox AHBS3 microscope coupled to an Olympus DP12 digital camera.
Cytotoxicity assay

Cytotoxicity was measured by assessing the cytolytic effect of bacterial extracts, as described previously (24), but using the MDTF (Mus dunni tail fibroblast) murine cell line as targets. Briefly, bacterial extracts obtained from *S. aureus* strains ATCC 15981 or V329 were diluted (1:10, 1:100 and 1:1000) in PBS and incubated (37°C, 5% CO₂) with 1×10⁵ MDTF cells (reaching confluence) /well in 24-well plates (BD Biosciences) for 90 min. PBS-treated cell extracts were used as control. After incubation, cells were rinsed with PBS, detached with trypsin, centrifuged (1000 × g, 5 min) and fixed with formalin. The number of live cells was determined by flow cytometry (FACS Scalibur, BD Biosciences).

**¹⁸F-FDG-MicroPET in vivo monitoring of biofilm infections**

Sealed catheters pre-colonized with either ATCC 15981 or V329 *S. aureus* strains were implanted subcutaneously in a total of 15 mice, as described above. An additional group of 15 mice carrying catheters pre-treated with TSB-glc alone (see above) was used as uninfected control (Fig. 1C). Results were assessed in 3 independent experiments (5 mice per strain and experiment). Following catheter implantation, infection was longitudinally evaluated *in vivo* (n=5) by ¹⁸F-FDG-MicroPET on days 1, 7 and 14. For this, fasted mice were anesthetized with 2% isoflurane in O₂ gas and intravenously injected with 18.8±1.9 MBq of ¹⁸F-FDG. Following 1 h of radiotracer uptake under continuous anesthesia inhalation, PET imaging was performed in a small animal tomograph (MicroPET; Mosaic, Philips, USA) by laying mice in prone position and capturing images for 15 min. Images were reconstructed using the 3D Ramla algorithm (a true 3D reconstruction) with 2 iterations and a relaxation parameter of 0.024 into a 128×128 matrix with a 1 mm voxel size, applying dead time, decay, random and scattering corrections. For ¹⁸F-FDG-uptake assessment, MicroPET images were analyzed using the PMOD
software (PMOD Technologies Ltd., Adliswil, Switzerland) and semi-quantitative results were expressed as the Standardized Uptake Value (SUV) index, obtained by normalization with the following formula: $\text{SUV} = \left[\frac{(\text{RTA}/\text{cm}^3)}{\text{RID}}\right] \times \text{BW}$, where RTA is the radiotracer tissue activity (in becquerels; Bq); RID is the radiotracer injected dose (in Bq) and BW is the mouse body weight (in grams). After qualitative inspection of the images, volumes of interest (VOI) were manually drawn on coronal 1-mm thick consecutive slices including the entire catheter area and axillary lymph nodes (ALN). For catheter image quantification avoiding manual bias of surrounding areas, a new VOI was generated semi-automatically using the threshold of 60% of maximum pixel for SUV mean calculation (SUV60 index).

Due to the small size of ALN, $^{18}$F-FDG-uptake was quantified and expressed as the mean of the 5 pixels with the highest uptake (SUVmax) included in the VOI selected (25).

$^{18}$F-FDG-MicroPET in vivo monitoring of antimicrobial therapy efficacy against S. aureus biofilm infection

Sealed catheters pre-colonized with S. aureus ATCC 15981 were implanted subcutaneously in 20 mice, as described above. From day 1 to day 7, 10 of these mice were treated daily with 0.1 mL of rifampin at 5 mg/mL diluted with PBS (0.5 mg/mouse of Rifaldin®, Sanofi-Aventis, France) by the oroesophagical route (Fig. 1D). The remaining 10 mice received PBS and were used as untreated controls. At days 1, 7 and 14 PI, a subgroup of mice (n=5) from both the untreated and the treated groups was monitored by $^{18}$F-FDG Micro-PET image analysis (see above). Also, at days 7 and 14 PI, the other subgroup (n=5) was used for determining the number of CFU/catheter. Moreover, resistance to rifampin was assessed in the isolated bacteria, using the standard MIC micro-dilution-broth method (26).

Statistical analysis

Kolmogorov-Smirnov test was first applied to assess the normal distribution of data.
Statistical comparison of means was performed by one-way ANOVA (CFUs data) or repeated measures ANOVA (Micro-PET data) tests, followed by the Fisher’s Protected Least Significant Difference (PLSD) test. Mathematical and statistical analyses were performed using StatView® Graphics for Windows (SAS Institute Inc).

**Results**

Clinical evaluation and in vivo kinetics of bacteria adhered to the surface of catheters implanted subcutaneously in mice

Signs of sepsis or discomfort were not found in any mice throughout the experimental period. Upon palpation, the majority of the animals with infected catheters had local subcutaneous inflammatory reactions on the back by day 7 PI, being stronger for strain ATCC 15981 compared to strain V329. The number of viable bacteria adhered to the surface of sealed catheters was in all cases around $0.8 \times 10^5$ CFU at the time of implantation, for both bacterial strains. Thereafter bacteria were quantified for a 49-day PI period (Fig. 1A), revealing that *S. aureus* ATCC 15981 persisted on catheters at high levels (around 6 log CFU/catheter) up to day 14 PI (Fig. 2). However, most (21 out 25) of the devices were naturally expelled before day 14. In contrast, all mice with *S. aureus* V329 infection maintained the catheters throughout the experimental period. In these mice, the number of viable V329 bacteria adhered to catheters increased moderately from days 1 to 3 PI, persisting thereafter at stable levels, around 4-5 log CFU/catheter (Fig. 2). Systematically, 1 of the 5 mice analyzed per experimental group receiving *S. aureus* V329 pre-colonized catheters was found free of infection from day 2 to the end of the experiment, indicating a 20% decreased success of catheter infection persistence in vivo when using this strain. Comparatively, in the in vivo successful infections by V329, catheters showed lower
(P<0.05) bacterial counts than those that became infected by ATCC 15981, at any time point till day 14 PI. Overall, these results indicated a clear strain difference in the infection kinetics in vivo regarding both, catheter expelling and number of live bacteria on the catheter.

**In vitro cytotoxicity**

To determine if strain ATCC 15981 would have higher cytotoxic capacity than that of strain V329, an in vitro cytotoxicity assay was carried out with bacterial cell extracts using MDTF cells as targets. Results revealed a 45% and 5% reduction of viable MDTF cells, respectively, confirming our hypothesis.

**Histopathology and immunohistochemistry**

The above observations on infection dynamics and cytotoxicity encouraged the study of inflammatory reactions, under the hypothesis that *S. aureus* ATCC 15981 and V329 infections would differ in the timing (kinetics) and strength of immune/inflammatory reactions. To assess this, a total of 33 animals (*S. aureus* ATCC 15981 and V329 infected groups, as well as uninfected control mice with uninfected catheters) was analyzed histologically by HE and IHC (Fig. 1B) after euthanasia in the area surrounding the catheter.

At day 1 PI, uninfected control mice had only a mild diffuse edema and few neutrophils surrounding the catheter area, both disappearing by day 7 PI (Fig. 3). This was compatible with unspecific inflammation subsequent to the surgical implantation of the catheter. In contrast, both bacterial biofilm infections induced prolonged focal and diffuse inflammatory responses, more intense in *S. aureus* ATCC 15981 than in V329 infections (Table 1). The focal reaction triggered by strain ATCC 15981 reached a maximum by day 7, and involved mainly neutrophils, fibroblasts, cell debris and some macrophages (Table 1
and Fig. 3). The reaction strength decreased by day 14 PI as a result of the natural expelling of catheters mentioned above (Table 1). Comparatively, strain V329 induced a delayed focal inflammatory reaction mainly characterized by cell debris (day 7) and macrophages (day 14), with no signs of remission.

Regarding the mouse connective capsule production, during the first week of infection strain ATCC 15981 induced a thin capsule that disappeared by day 14, accompanied by catheter expelling. In contrast, the capsule induced by strain V329 appeared at day 7 and became moderately thick by day 14 PI (Table 1).

In both infections, the diffuse inflammatory reaction observed at day 1 PI in the area surrounding the catheter had more neutrophils than the uninfected controls. Thereafter, fibroblasts appeared by day 7 in both bacterial infections, and remained in moderate numbers in strain V329 infection (Table 1).

As expected from the nature of the biofilm matrix and the specificity of the anti-matrix-polymer antibodies used, the anti-Bap and anti-PIA/PNAG antibodies stained specifically free bacteria, bacterial aggregates, and neutrophil-phagocytized bacterial antigen in *S. aureus* strain V329 and ATCC 15981 infections, respectively (Fig. 3). Strikingly, anti-PIA/PNAG antibody, and not anti-Bap, stained the cytoplasm of foam cells disseminated far away from the catheter, between the muscular and adipose tissues in the ATCC 15981 but not in V329 infection (Fig. 3). Uninfected animals did not yield positive reactions with anti-PIA/PNAG or anti-Bap antibodies.

**In vivo infection monitoring by MicroPET**

Knowing the above strain differences, we determined if both *S. aureus* infections could be assessed and would differ *in vivo* by MicroPET imaging analysis, according to the experiment outlined in Fig. 1C. Unlike uninfected controls, $^{18}$F-FDG was increased in both,
catheter (Fig. 4A) and ALN (Fig. 4B), areas of infected mice.

In the catheter area (Figs. 4A and 4C left panel), $^{18}$F-FDG uptake was detected as soon as day 1 PI and remained stable for each bacterial strain, without statistical differences in the mean SUV60 index values for a given strain along the study period. In addition, strain differences in favor of ATCC 15981 were observed at days 7 and 14 PI.

In ALN (Fig. 4B; and Fig. 4C right panel), SUVmax values were very low or null at day 1 PI, increasing significantly by day 7 PI in infections by ATCC 15981 and in a delayed and decreased manner (by day 14 PI), in those by V329. Thus, differences between days 1 vs. 7 or 14 PI was highly significant ($P \leq 0.0005$) in ATCC 15981 infection, but it was only a trend ($P = 0.08$) in $S. aureus$ V329 infection. Overall, $^{18}$F-FDG uptake was detected in the catheter area earlier than in ALN, but in ALN the signal remained at high levels after catheter expelling.

Altogether, the results obtained indicate that the early (day 7) and strong $^{18}$F-FDG uptake observed in ATCC 15981 infections (Fig. 4) corresponded to the early and strong inflammatory reaction (Fig. 3) and increased cytotoxicity triggered by this strain compared to strain V329 infections. Also, in V329-infected mice, the steady increase in ALN SUVmax index corresponded to the steady increase in focal subcutaneous inflammatory reaction (Fig. 3).

$^{18}$F-FDG-MicroPET image analysis for evaluating rifampin treatment efficacy

The strong and stable $^{18}$F-FDG uptake in $S. aureus$ ATCC 15981 infections suggested that this strain was a good choice as a prototype to determine the utility of the $^{18}$F-FDG-MicroPET mouse model for monitoring in vivo the efficacy of antibiotic treatment. To assess this, $^{18}$F-FDG uptake was measured 24 h after implantation of the catheter pre-colonized with $S. aureus$ ATCC 15981 in a group of 10 mice, which were subsequently
submitted to antimicrobial therapy with rifampin (Fig. 1D) and the results compared to those obtained in 10 untreated control mice having infected catheters. As shown in Fig. 5, the SUV60 and SUVmax values obtained in the untreated group were very close to those obtained in the previous experiment (Fig. 4). The SUV60 values in the catheter area (Figs. 5A and 5B, left panels) decreased significantly by day 7 in the rifampin-treated group. This decrease was accompanied by a decrease at day 7 in the number of CFU/catheter (Fig. 5C). Also, treated animals maintained the catheter beyond day 14, this being a sign of controlled infection.

However, an increase in SUV60 index was observed in some rifampin treated animals by day 14 PI compared to day 7. This fluctuation of SUV60 values from days 7 to 14 was likely due to the appearance of rifampin resistant variants of the *S. aureus* ATCC 15981 strain, as verified by growth in the presence of rifampin (16 μg/mL).

Finally, the SUVmax values in ALN of rifampin treated mice were null at day 7 (Fig. 5A and 5B, right panels) and increased slightly at day 14 PI (Fig. 5A right panel).

**Discussion**

The utility of an innovative $^{18}$F-FDG-MicroPET *in vivo* imaging technology in *S. aureus* biofilm infections was assessed in a sealed-catheter mouse model developed in this work. This mouse model was found useful for (i) monitoring long term *S. aureus in vivo* infections in a same animal; (ii) detecting *S. aureus* strain differences in infection dynamics and pathogenicity; and (iii) evaluating the efficacy of antimicrobial therapy, using unlabeled bacteria.

To our knowledge, this is the first work using $^{18}$F-FDG-MicroPET image method for diagnosing and monitoring bacterial biofilm evolution in mice. This method has been
successfully applied for diagnosing clinical inflammatory processes, especially those involving neutrophils and macrophages (27, 28). However, bacteria may also uptake $^{18}$F-FDG \textit{in vivo}, knowing that PIA/PNAG dependent \textit{S. aureus} biofilm production \textit{in vitro} may be enhanced by glucose supplementation (TSB-glc here). Unfortunately, the technique did not distinguish the signal exclusively emitted by bacteria from that generated by inflammatory cells. $^{18}$F-FDG-MicroPET has also been used in animals, specifically in rabbit staphylococcal osteomyelitis models (18, 19). The mouse model, which involved the use of sealed catheters and required one-step rather than two-step (10, 11) surgical procedure in the absence of a cement block, simplified the assessment of direct host-pathogen interactions. In line with our observations, in a rabbit model detecting bone staphylococcal infections, a direct relationship was found between PET image intensity, degree of leukocyte infiltration and increased virulence (in \textit{S. aureus} compared to \textit{S. epidermidis}) (18). However, detection of \textit{S. aureus} strain differences and antibiotic monitoring was not attempted.

Both \textit{S. aureus} strains involved in our study were strong biofilm producers to guarantee a successfully catheter pre-colonization and subsequently attempt chronic infection in mice. The SUV image differences detected \textit{in vivo} between bacterial strains were in consonance not only with those found in infection kinetics and cytotoxicity differences, but also with strain-associated differences in bacterial dissemination and the triggered histological changes. The strain differences in the \textit{in vivo} colonization capacity may be attributed to the defective primary binding of V329 Bap-synthesizing bacteria via host molecules (29, 30) and to the tight cell-to-cell Bap mediated bonds compared to PIA/PNAG mediated binding (ATCC 15981 biofilms). The decreased inflammatory reaction triggered by V329 compared to ATCC 15981, can be explained by the decreased number of bacteria adhered to the
catheters in vivo, the diminished cytotoxicity and the natural replacement of toxin genes by the bap gene in strain V329 (1, 23). On the contrary, the intense inflammatory reaction triggered by ATCC 15981 was expected as this clinical otitis isolate that encodes exocellular proteases (21), had a high colonization capacity and was highly cytotoxic, all of which may have triggered the expulsion of infected catheters.

The finding that the $^{18}$F-FDG-MicroPET uptake in untreated mice was delayed in the draining ALN compared to the catheter area was compatible with the development of an adaptive immune response in the ALN within a two week period following the immune response at the initial infection site. This is in agreement with a previous study evaluating S. aureus contaminated muscle injury in rabbits through histological and microbiological analysis (31), in which the draining lymph node provided a delayed signal compared to that of the originally infected body site. Altogether, these observations outline the utility of draining lymph node $^{18}$F-FDG-MicroPET imaging in chronic infections.

Biofilm-embedded bacteria are tenacious and 100 to 10,000 times more resistant to antimicrobial treatment than non-embedded bacteria, even in vitro (32, 33). Different studies show that treatment with high doses of rifampin is effective against S. aureus biofilm bacteria in vitro (33-35) and in vivo (12), but it leads also to the appearance of rifampin resistant mutants in the rpoB gene, and consequently to increased bacterial counts and optical imaging signal, as observed in studies on natural human device infections (36) and mouse intra-venous catheter infections using lux labeled bacteria (10, 13). Thus, the appearance of resistant variants, verified in vitro, was not only expected but also advantageous in our model since it allowed the detection of variation in $^{18}$F-FDG-MicroPET uptake intensity along treatment. Finally, the absence of catheter expelling and of PET image signal in ALN upon antibiotic treatment could be indicative of antibiotic
efficacy and pointed out the value of ALN to assess immune response and infection progress.

The $^{18}$F-FDG-Micro-PET mouse model allowed follow-up individual studies, reducing the number of euthanized experimental animals, in line with the current international guidelines on animal welfare. A promising near-infrared fluorescence imaging mouse model using an alternative pre-colonized device (polymer disk) to detect inflammation (reactive oxygen species produced by inflammatory cells) upon implantation has been developed recently (14). The utility of this model to assess differences in pathogenicity between bacterial strains and to monitor the efficacy of antibiotic therapy has not been reported so far.

Overall, the model was highly reproducible; of note, the *in vivo* follow up studies revealed a low intra-group or inter-experiment variability. Also, the model was cost-effective, and could be suitable for preclinical studies, as it could yield useful data to diagnose *S. aureus* biofilm infections and to develop, improve and monitor single and combined antimicrobial treatments. Finally, the model could be successfully employed to assess *in vivo* differences between natural isolates and/or genetically modified bacteria in virulence/pathogenicity and to investigate host-pathogen interactions of clinical relevance in device associated and biofilm infections.

**Acknowledgments**

This work was supported by grants from Gobierno de Navarra “IIM13002.RI1” and MICINN “CIT-010000-2009-32“. We thank Dr. Ramsés Reina (Instituto de Agrobiotecnología, Pamplona, Spain) for cell culture, Dr. Gerry Pier (Harvard Medical School, USA) for anti-PIA/PNAG antibody and Dr. I. Lasa and his group (Instituto de
Agrobiotecnología, Pamplona, Spain) for anti-Bap antibody and support on technical approaches.

References


**TABLE 1.** Inflammatory reaction observed with haematoxylin and eosin (HE) staining, after subcutaneous implantation of sealed catheters in mice.

<table>
<thead>
<tr>
<th>Inflammatory reaction</th>
<th>S. aureus strains used in catheter colonization and days post-implantation</th>
<th>Uninfected controls</th>
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<tbody>
<tr>
<td></td>
<td>ATCC 15981</td>
<td>V329</td>
</tr>
<tr>
<td>Presence of implant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Focal</td>
<td>Cell debris</td>
<td>++</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Macrophages</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Fibroblasts</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>Size of capsule</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Diffuse</td>
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<td>Neutrophils</td>
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<td>Fibroblasts</td>
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**d, day post-implantation.**

* All mice infected with strain ATCC 15981 had expelled the catheters by this time and data obtained then refer to these mice that had already lost the implants. Number of animals/group/assessment day: n=5 for each of the infected groups and n=1 for uninfected controls.

-, Absent to very low; +, Low; ++, Moderate; ++++, Strong; ++++, Very strong.
Figure Legends

**Figure 1.** Design of four experiments according to animal group distribution and time-points for analysis (indicated with arrows): a) Bacteriological study on the evolution of infections (log_{10} CFU/catheter); b) histological changes in subcutaneous tissue during infection, using haematoxylin-eosin (HE) and immunohistochemical (IHC) staining; c) *in vivo* {^{18}}F-FDG-MicroPET imaging in an infection follow up study; and d) {^{18}}F-FDG-MicroPET imaging and bacteriological study (log_{10} CFU/catheter) in mice carrying infected catheters and then treated (T) daily for one week with rifampin (0.5 mg/day/mouse) or receiving PBS (untreated controls). Experiments (a), (b) and (c) were each carried out independently with strains ATCC 15981 and V329 and also included animal groups with uninfected catheters (control groups). Experiment (d) was done with strain ATCC 15981.

**Figure 2.** Quantification (mean ± SD; n=5) of viable *S. aureus* ATCC 15981 and V329 bacteria (CFU) found on infected sealed catheters pre-colonized and implanted subcutaneously in mice. Catheters infected with strain ATCC 15981 (close squares) were naturally expelled between days 7 and 14 post-implantation (PI). At each interval, 1 out 5 catheters successfully pre-colonized *in vitro* with strain V329 became uninfected after subcutaneous implantation *in vivo*, and the corresponding data have not been included in the figure. * Statistical comparisons by Fisher’s LSD test: P<0.05 between both bacterial strains, at days 1, 7 and 14.

**Figure 3.** Histological study on *in vivo* biofilm infections in subcutaneous tissues surrounding the catheter, stained with hematoxylin-eosin (HE) (A-G) or immunohistochemistry (IHC) (H-I) techniques. Sealed catheters pre-colonized with *S.
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(C) log CFU/catheter, obtained at day 7, when differences between groups became more evident and statistically significant (*$P<0.05$; **$P<0.005$). Panel B: MicroPET images of representative untreated and treated mice, in dorsal (left images) or ventral (right images) positions showing the catheter area (arrow) and ALN (arrowheads), respectively; (C): box-plots representing the number of bacteria (log CFU/catheter) adhered to the surface of sealed catheter in untreated (dark boxes) and rifampin treated (striped light boxes) mice.
TABLE 1. Inflammatory reaction observed with haematoxylin and eosin (HE) staining, after subcutaneous implantation of sealed catheters in mice

<table>
<thead>
<tr>
<th>Inflammatory reaction</th>
<th>S. aureus strains used in catheter colonization and days post-implantation</th>
<th>ATCC 15981</th>
<th>V329</th>
<th>Uninfected controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1d</td>
<td>7d</td>
<td>14d</td>
</tr>
<tr>
<td>Presence of implant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell debris</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Size of capsule</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Edema</td>
<td></td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
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<td>-</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

d, day post-implantation.

da All mice infected with strain ATCC 15981 had expelled the catheters by this time and data obtained then refer to these mice that had already lost the implants. Number of animals/group/assessment day: n=5 for each of the infected groups and n=1 for uninfected controls.

-, Absent to very low; +, Low; ++, Moderate; +++, Strong; ++++, Very strong.
Days post-implantation:

A

ATCC 15981 (50 mice)
V329 (50 mice)

B

ATCC 15981 (15 mice)
V329 (15 mice)
Uninfected (3 mice)

C

ATCC 15981 (15 mice)
V329 (15 mice)
Uninfected (15 mice)

D

ATCC 15981 (20 mice)

Histology of subcutaneous tissues of infected (n=5) and uninfected (n=1)

Three independent experiments (n=5)

(10 mice treated and 10 mice untreated)
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