Evaluation of four experimental osteomyelitis infection models by using precolonized implants and bacterial suspensions

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ABSTRACT – Staphylococcus aureus osteomyelitis, a major problem in orthopedic surgery, often involves biofilm bacteria adhering to implants and surrounding bone and tissues. The inadequacy of therapy or immunological surveillance has encouraged studies using animal models which simulate natural osteomyelitic infections, ensure the development of infections and avoid mortality. We evaluated 4 models for infection (8 animals/model) in rats, using stainless-steel implants in tibiae and a very adherent slime-producing bacterial strain. Each animal received: an implant containing a 12 h-biofilm with about 10^6 cfu (Model 1); an implant containing this biofilm and a suspension with about 10^4 cfu (Model 2); a sterile implant and a suspension with about 10^5 cfu (Model 3); or a sterile implant and a suspension with about 10^6 cfu (Model 4). 63 days after surgery we found 100% rat survival, colonization of bone by implant biofilm bacteria in some animals and local, but not systemic infections. Model 1 (but not Models 2–4) reproduced an infection in both, tibiae and implants, most reliably (in 100% of the animals). Model 3 was the least reliable ($p < 0.01$, 25% infected implants, 12% infected tibiae).

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Staphylococcus aureus, the commonest pathogen in hospital-acquired infections (McKenney et al. 1999), is also the main cause of osteomyelitis. Both S. aureus and Staphylococcus epidermidis are frequently associated with implant infections (Hanssen and Rand 1998) and are the chief infectious agents in prosthetic joints. These bacterial species can produce exopolysaccharides (slime), mediating bacterial adherence and biofilm formation on various surfaces (Costerton et al. 1999, McKenney et al. 1999). Biofilms that adhere to implants and surrounding tissues are difficult to eradicate with phagocytosis (because of their size) or antibiotic treatment, and therefore induce chronic infections (Mayberry-Carson et al. 1984, Gristina 1994, Amorena et al. 1999). This problem has encouraged research in this field. Particularly, in the field of human osteomyelitis, animal models have been sought to reproduce the processes involved in this disease. After the first S. aureus infection model was developed (Rodet 1973), the disease was reproduced in rabbits (Andriole et al. 1974, Mayberry-Carson et al. 1984, 1992, Norden and Budinsky 1990, Lambe et al. 1991, Lasierra et al. 1994) and rats (Power et al. 1990). Infection has been reproduced by systemic inoculation of bacterial suspensions (Lasierra et al. 1994) or direct introduction of bacteria at the site of the implant, in suspension (Lambe et al. 1991, Mayberry-Carson et al. 1992, Lasierra et al. 1994), in suspension and in biofilm (mixed models; Barth et al. 1989, Gracia et al. 1998) or in biofilm alone (Ward et al. 1992).

A common problem in some of these in vivo models is that the success in reproducing osteomyelitis is lower than expected, leading to the misuse of animals and inefficiency of the model for comparative purposes in therapeutic, prophylactic, immunologic or physiologic studies. Other problems are related to the nature of the bacterial isolate used for infection, some bacteria being eliminated by the immune system more easily than others with a high capacity for biofilm production (Gracia et al. 1998). A few models use a sclerosing agent to

promote infection (Kaarsemaker et al. 1997), but the agent may make it difficult to reproduce the natural osteomyelitis process. Finally, some mixed infection models which may have high infection rates (Gracia et al. 1998) can probably be simplified by using one of the components—i.e., bacterial suspension or a biofilm.

We studied 4 different experimental osteomyelitis infection models in rats, based on the use of a highly adherent slime-producing (SP) S. aureus isolate and a stainless steel implant (sterile or precolonized with bacteria of this isolate), to determine which of these models more reliably reproduces a chronic (9 weeks) osteomyelitis infection with no increase in the mortality rate.

Material and methods

Microorganism. We used a very adherent slime-producing isolate of S. aureus (strain 9213 SP). This was produced in our laboratory from the original non-slime-producing (NSP) isolate by selecting adherent colonies, as described elsewhere (Baselga et al. 1993). SP isolates could be distinguished from NSP isolates by the morphology of their colonies in Congo red agar (Freeman et al. 1989, Baselga et al. 1993), being rough or smooth with SP and NSP isolates, respectively. SP cells also adhered better to inert surfaces than NSP cells (Christensen et al. 1985, Baselga et al. 1993).

Ribotyping (using the enzyme Eco R1), antibiotic susceptibility testing of bacteria in suspension (using the microdilution procedure of the National Committee for Clinical Laboratory Standards, NCCLS 1990) and determination of colony morphology in Congo red agar were used to ensure that in the animal model used, the SP isolate recovered from implants and tissues at the end of the experiment (63 days after surgery) was the same as that originally used to initiate the infection.

Implants and biofilm formation. Using a previously described procedure (Gracia et al. 1998), biofilms were formed on 1.5 cm long stainless steel pieces, obtained by sectioning 18 G needles. Briefly, after autoclave-sterilization, pieces (apart from those introduced sterile into the animal) were placed in tubes containing 2 mL of growth medium (tryptone soy broth, TSB, Difco, Detroit) and 50 μL of a stationary bacterial culture was added to each tube. Biofilms were developed on the metal pieces for 12 h at 37 °C. Pieces provided with biofilms were removed with tweezers under sterile conditions. After washing three times with phosphate buffered saline (PBS) to discard unbound bacteria, implants were stored at 4 °C for no more than 1 h to avoid changes in the number of bacterial cells before surgery. Before the in vivo infection, the number of bacteria forming a biofilm was determined in 6 control pieces which were not used for implantation. For this purpose, implants were resuspended in 2 mL of PBS. After 30 min sonication at 40 Hz (22–24 °C) to disintegrate bacterial aggregates and detach implant-bound bacteria, viable microorganisms were plate-counted on tryptone soy agar (TSA, Difco, Detroit), each 12 h-colonized implant having 6.15 (SD 0.12) $\log_{10}$ colony-forming units (cfu).

Preparation of bacterial suspensions. To prepare the inoculum of bacterial suspensions used at the site of infection, bacteria were grown for 24 h in TSB and the concentration adjusted by spectophotometry (A_{540}) thereafter to 10^5, 10^6 and 10^7 cfu/mL of PBS, depending on the animal infection model used. Each animal received 0.1 mL of the suspension (about 10^4, 10^5 and 10^6 cfu, respectively).

Animals. 32 Wistar male rats having an average weight of 375 g were used. Rules established in The National Research Council's guide for animal experimentation were followed for animal handling and care. The rats were divided into four groups (8 animals/group) according to the infection model (Table). There was homogeneity between the groups as regards body weight at the beginning of the experiment. Each animal received: only an implant containing a 12 h biofilm (Model 1); an implant containing a 12 h biofilm and a bacterial suspension of about 10^4 cfu (Model 2); a sterile implant and a bacterial suspension of about 10^5 cfu (Model 3); or a sterile implant and a bacterial suspension of about 10^6 cfu (Model 4). On the day of surgery, implants (colonized or sterile, according to the experimental model) and bacterial suspensions (when indicated) were placed in the left tibia of the animal, previously anesthetized with an intramuscular injection of 50% ketamine, 40% diazepam and 10% atropine. After surgery, ani-

mals were examined daily for signs of inflammation, induration, pain, fever and weight loss. Infection was allowed to persist for 63 days. Blood was obtained by cardiac puncture before killing the animals with potassium chloride. Tissue samples were obtained post-mortem.

Surgical procedure. After shaving and disinfecting the skin of the surgical zone surrounding the rotula, an incision was made, drawing back the articular fascia to approach the rotulian ligament with a scalpel. The tibia was drilled from the proximal to the distal area using an 18 G needle (1.2 × 40 mm), and the metallic implant (containing the preformed biofilm, as specified) was introduced into the resulting cavity. When indicated, bacterial suspensions were inoculated into the implant site. The skin was disinfected after closing the incision with sterilized staples.

Assessment of the infection. An infection was confirmed at the end of the experiment by radiographs, bacterial analysis (plate count in TSA) and histologically (in tissue sections 4 μm thick). Before Gram staining for histological examinations, samples were decalcified in EDTA-PVP for 26 days. After washing, the samples were consecutively dehydrated in different concentrations of alcohol (70%–80%–96%–100%–100%) and xylol. Thereafter, they were placed in paraffin. The bacterial analyses were done at the end of the experiment (9 weeks after surgery), tibiae containing implants, tibiae from the other leg of the animal (not operated on, but used as a control to determine whether the infection had been systemically transmitted to this leg) and samples of liver, spleen and whole blood (in EDTA) were taken from all the animals. A 30 min sonication (40 Hz at 22–24 °C), which did not significantly affect the number of viable bacteria, had to be done to determine the number of bacteria on the implant (plate count).

Transmission electron microscopy (TEM). TEM was used to show the homogeneity of biofilms formed on implants at various stages of bacterial growth (12 h- and 48 h-biofilms were studied with TSB as the growth medium, see above), with the following specifications. Before biofilm formation, 500 mL of stationary growth phase bacterial cultures of isolate 9213 SP were added under aseptic conditions, together with 4.5 mL of growth medium (TSB) to a 60 mm^2 stainless steel surface. Biofilms were developed in 12 or 48 h. For TEM analysis, 2.5 mL of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7), containing 0.05% ruthenium red, was added following three washes with PBS. After 2 h at 22–24 °C, samples were washed several times with PBS and dehydrated in a sequential series of 50%, 70%, 80% and 100% ethanol (10 min each). To separate the biofilm from the supportive material, 100% ethanol was added and biofilms were sectioned into 3 × 3 mm^2 square pieces with a scalpel. After discarding ethanol, adding propylene oxide, and applying a mild agitation, the sample squares were placed in a mixture of propylene oxide and resin (Sigma, Madrid, Spain) in a proportion of 1/1 (vol:vol) for 12 h at 22–24 °C. Samples were then transferred to 100% resin. After 2–3 days at 22–24 °C, they were transferred into a newly made resin for 16 h at 70 °C and then sectioned for TEM examination.

Scanning electron microscopy (SEM). SEM, together with histological studies, was done according to standard procedures to assess infection in rat tibia and tissue around the precolonized implant 63 days after surgery. The sample, which had been in PBS for 17 days, was fixed with 4% glutaraldehyde and postfixed with osmium tetroxide (OsO_4); drying by lyophilyzation and application of gold were then done.

Statistics. A chi-square test was used to compare the percentage of infections at the end of the experiment in the various infection models. Tibiae and implants were analyzed separately.

Results

Transmission electron microscopy, performed on metal-associated biofilms (Figure 1), showed biofilm homogeneity throughout the supportive surface during biofilm formation, as regards thickness and distribution of bacterial cells. This homogeneity permitted comparisons between animal groups (Table). At the end of the experiment, the animals were alive, had not lost weight (all weighed about 475 g), were clinically healthy and had completely recovered their leg movements. Bacteria were recovered only from operated tibiae and implants.

In all groups about 10^5 bacteria were recovered from implants and 10^3 bacteria from tibiae, with

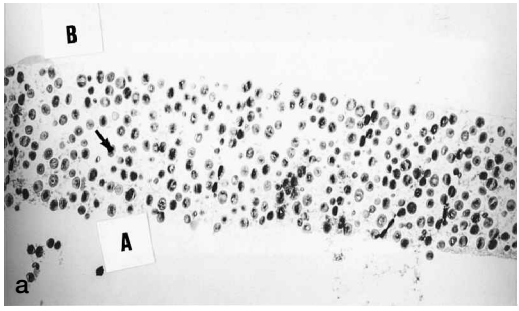
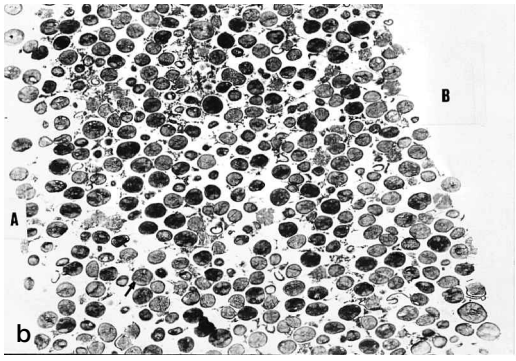


Figure 1. Transmission electron microscopy (TEM) micrograph of biofilms developed for 12 h (a) and evolution of this biofilm when growth occurs for 48 h (b). A—biofilm limit line in contact with the culture medium. B—biofilm limit line in contact with the implant. Arrow indicates a bacterial cell. Magnification ×450 (a) and ×3800 (b).



Note the homogeneity of the biofilm during formation.

Comparison of Staphylococcus aureus infection rates in 4 rat osteomyelitis infection models which differed in the number and state (suspension vs. biofilm) of microorganisms used in the bacterial inoculum

Experimental model (n 8/model)	Log_{10} cfu introduced at surgery [b]	Percentage of infected animals 63 days after surgery [a]	
		Implants	Tibiae
1. Colonized implant	6.15	100	100
2. Colonized implant + bacterial suspension	6.15 + 4	100	62 [c]
3. Sterile implant + bacterial suspension	0 + 5	25 [d]	12 [e]
4. Sterile implant + bacterial suspension	0 + 6	75	75

[a] Results are percentage of animals. The mean and standard deviation of the number of bacteria recovered from implants and tibiae were similar among various groups (about 10^5 and 10^3 bacteria, respectively); no significant differences in infected animals at the end of the experiments were found.
[b] Since the number of bacteria is given in logarithmic units, there is hardly any difference in the number of bacteria in groups 1, 2 and 4 (about 0.1 log cfu), all three groups having received about 1 million bacteria.
Probability that the percentage differs from 100%, using the chi-square test.
[c] $p = 0.08$
[d] $p = 0.004$
[e] $p = 0.002$

similar standard deviations in the various groups and no significant differences between infected animals, independently of the group to which they belonged. Septicemia was not detected in any of the experimental groups, since the liver, spleen, blood and control tibiae (which had not undergone surgery) had no bacteria, indicating that the infection had not spread, but was confined to the site of surgery. The recovered bacteria had the same ribotype, colony morphology in Congo red agar (rough) and antibiotic susceptibility profile as that of the bacterial inoculum used for infection, disregarding the possible presence of contaminating microorganisms at the site of infection. Colonization of tibiae, as judged by the site of bacterial cell count (plate count), took place in the rat tibia by bacteria shed from the implant biofilm. This is shown by SEM (Figure 2).

Once they had adhered to bone, bacteria grew, forming biofilms bound to trabecular bone, as seen with Gram staining (Figure 3). Radiological studies showed that all infected tibiae had a periostial reac-

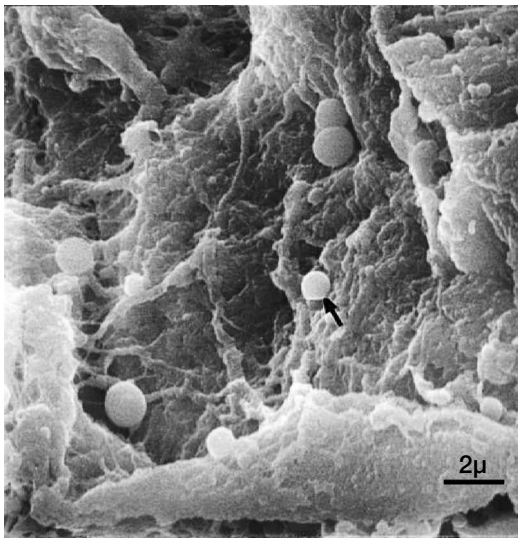


Figure 2. Scanning electron microscopy (SEM). Micrograph of an infected rat tibia showing the migration of bacteria shed from the implant biofilm surface via the trabecular bone. The rat was infected at surgery by a colonized implant (group 1). Arrow indicates a bacterial cell. Magnification ×5000.

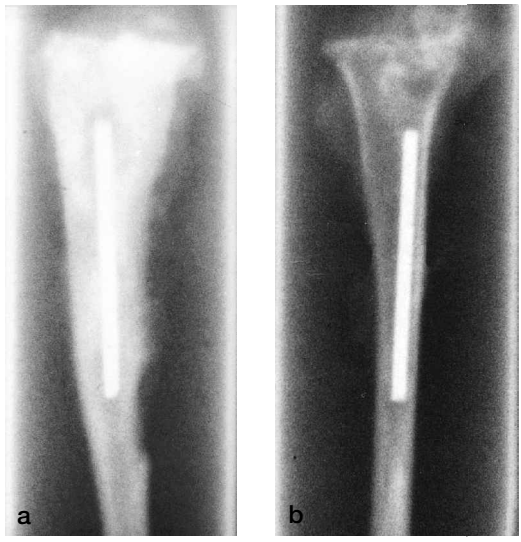


Figure 4. Radiographs of rat tibiae provided with implants. Staphylococcus aureus experimental osteomyelitis in tibia showing a moderate periostial reaction around the implant and well-defined osteolytic areas (a) and uninfected tibia (b).

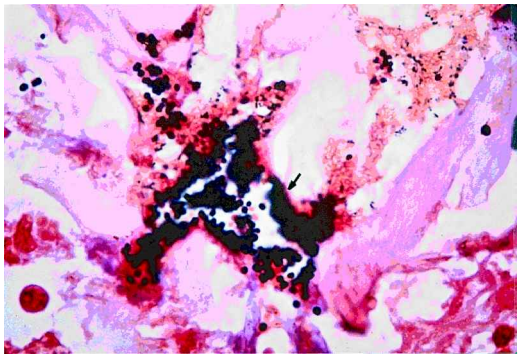


Figure 3. Gram-stained histological preparation of a trabecular bone section from a tibia with chronic osteomyelitis. Staphylococcal biofilms that have adhered to bone are shown (arrow). The rat was infected at surgery by a colonized implant (group 1). Magnification ×1000.

tion around the implant and well-defined osteolytic areas, but healthy tibiae had a uniform and normal distribution of bone in this area (Figure 4).

The success in inducing an infection varied among experimental infection models, as shown by the percentages of animals in which the tibia and/or the implant became infected (Table). Only the model having a colonized implant with no bacterial suspension inoculum (Model 1) reliably (in 100% of the animals) induced an infection in both tibiae and implants. Model 2 ,with an inoculation of 10^4 bacteria in suspension and a colonized implant, reliably reproduced an infection (100%) in implants, but was less successful ($p < 0.05$) in tibiae. In the two models in which the infection was induced solely by a bacterial suspension with a sterile implant, the percentage of success in establishing the infection in implants and tibiae also appeared to decline, especially when the inoculum was reduced from 10^6 (Model 4) to 10^5 bacteria (Model 3). The latter model significantly lowered the likelihood of an infection ($p < 0.05$) in both, implants and tibiae, and had the lowest success rate of the models studied (only 2 implants and 1 tibia were still infected at the end of the experiment).

Discussion

We found among the models clear differences in reliability to reproduce a persistent osteomyelitis in rats. The implant precolonized with a 12-h biofilm (about 10^6 cfu), having a highly adherent isolate, but no bacterial suspension inoculum, very efficiently induced an infection in the implant and bone for over 2 months, and did not lead to weight loss or death. The selection of the bacterial isolate

used for the implant before colonization and bone infection is of particular importance for establishing osteomyelitis. SP strains strongly adhere to various biomaterials and resist phagocytosis or antibiotic treatment in vitro, colonize tissues, resist antibiotics and cause chronic infections in vivo (Baselga et al. 1993, Gracia et al. 1997, Monleón et al. 1997, König et al. 1998, Amorena et al. 1999). Such biofilm-associated problems have contributed to the use of this type of strains in chronic disease models (Deighton et al. 1996, Gracia et al. 1997, Hanssen and Rand 1998, König et al. 1998).

The in vivo evidence for biofilm formation shown with Gram staining of the SP strain used in this study strongly suggests that this isolate produces biofilms not only in vitro, but also in vivo, despite a corresponding change of the growth medium in both situations (from TSB to the host internal environment). The findings that the infection persisted (for at least 2 months) is encouraging, considering that murine species can resolve an infection by a very active immune response (Miller et al. 1987, García-Álvarez et al. 1997, Navarro-Zorraquino 1997).

Another way to promote bacterial attachment is to use implant/surfaces coated with specific host molecules, such as fibronectin, and S. aureus strains which easily bind these molecules via receptors in the bacterial cell wall (Domingue et al. 1994, Fischer et al. 1996). However, some SP strains heavily coated with exopolysaccharides (slime) may find it difficult to interact with the host proteins via these cell wall receptors (Baldasarri et al. 1997). In the absence of phase variation (from SP to NSP), host molecule coating of tibiae would inhibit the colonization of tibiae by SP cells.

An alternative approach to the use of precolonized implants to facilitate the development of osteomyelitis is the application of a sclerosing agent (Norden 1970, Crane et al. 1977, Rinsky et al. 1983, Mayberry-Carson et al. 1984, Lasierra et al. 1994). Persistence of an infection has been observed in a rabbit model, using a sclerosing agent, in which a stable biofilm was found from 1 to 8 weeks after bacterial inoculation at the implant site (Belmatoug et al. 1996). However, the ability of this agent to change the nature of the interactions between bacteria and host directly or indirectly cannot be disregarded. Changes in hydrophobicity caused by abrogation of slime production are known to alter bacterial attachment and biofilm formation (Baldasarri et al. 1997).

Unexpectedly, in our study, the model including the precolonized implant and inoculation of a bacterial suspension having a low count (10^4 cfu) had only a 62% infection rate in tibiae. This low success rate may be due to the greater capacity of a low count bacterial inoculum to stimulate the organism's immunological response, thereby reducing colonization of the tibia by bacteria shed from the implant biofilm. More immunological studies are warranted to elucidate this finding. An increase in the number of biofilm bacteria in the biofilm coating the implant (10^8 rather than 10^6 cfu) and a change in the biofilm characteristics (growth in TSB supplemented with glucose rather than unmodified TSB) may explain the apparent discrepancy between the findings in this study and those in our previous mixed model study (Gracia et al. 1998), which had a 100% infection success rate.

In that study (Gracia et al. 1998), preliminary data on comparatively few animals suggested that the use of only a bacterial SP suspension (10^4 bacteria per inoculum) at the site of surgery and no sterile implant might be a promising method for reproducing ostemyelitis. However, in the present study involving the inoculation of 10^6 bacteria and use of a sterile implant in more animals, the success rate of bone infections (group 4 here) appeared to decrease to about 75%. We do not know whether in future work use of a higher number of bacteria in suspension (10^7 rather than 10^6 bacteria) and/or the absence of a sterile implant at surgery would have a 100% infection success and animal survival rate, comparable to Model 1.

In vitro biofilm models may help us to understand the processes involved in osteomyelitis, but the information obtained from such models is incomplete. In the implanted individual, changes produced at the site of infection are bacteria- and host-dependent. These changes affect the metabolism, size and density of bacteria, the nature of bacterial products in the biofilm and the outcome of the infection. They may alo be related to local nutritional restrictions (Bergamini et al. 1994) or immune surveillance phenomena (Pérez Fernández et al. 1995, Gracia et al. 1998). Unfortunately,

antibodies cannot facilitate the opsono-phagocytic elimination of bacteria in biofilms (Costerton et al. 1999). Immunodeficiency diseases, immunodepression stages, diabetes, malnutrition, obesity and concurrent infections may also affect the fate of osteomyelitic infections (Navarro-Zorraquino 1997, Hanssen and Rand 1998). The overall complexity of these mechanisms stimulates the development of in vivo osteomyelitis models.

Animal models based on systemic infections induced by bacterial suspensions (Deysine et al. 1983, Lasierra et al. 1994) are comparable to infections at various sites in the body, responsible for subsequent bone colonization with hematogenous transmission of bacteria. However, in these models, the success rate for development of osteomyelitis may be low and the systemic infection may increase the mortality. On the other hand, models using a precolonized implant mainly approach the situation of implanting contaminated material or use of a non-sterile procedure at surgery. Several of the local infection models developed so far have been based on use of a bacterial suspension together with implantation of sterile metal pieces at the site of infection (Andriole et al. 1974, Power et al. 1990, Widmer et al. 1990, Lasierra et al. 1994) or sterile intramedullary silicone catheters in tibiae (Mayberry-Carson et al. 1992), but they do not always cause a persistent infection. To improve infection rates, precolonized implants have been used alone with intra-peritoneal implantation (Ward et al. 1992), or together with a bacterial suspension in orthopedic surgery models (Barth et al. 1989, Gracia et al. 1998). However, in these mixed models, the effect of the biofilm bacteria cannot be easily distinguished from that of the bacterial suspension used to initiate the infection and difficulties arise in aseptically and reliably introducing a bacterial suspension into the site of the implant.

In vitro precolonized implants, which are subsequently introduced in vivo, may have the advantage of ensuring the presence of bacteria which cannot be phagocytosed in the host and providing homogeneity among animals in the number of cells and biofilm size (multilayers) initially present at the start of the infection, as shown here with TEM. This homogeneity may be very useful in the subsequent application of these biofilms, for example, in comparative studies of antimicrobial chemotherapy. In contrast, irregular biofilms may form when biofilms are initiated in vivo. Although this situation would be closer to that occurring in human osteomyelitis, comparative studies in animal models would be jeopardized by the large differences in biofilm development between animals, because of variations in the access of bacteria to different zones in the implant and tibia, access of nutrients and immunological or physiological microenvironments.

An experimental osteomyelitis model must reproduce an infection not only for a long time, but also in a high percentage of animals. It should also ensure a low mortality rate. The information provided in this study may prove useful in reproducing S. aureus osteomyelitis according to these criteria. It may also be applicable to studies on therapy and immunological methods of controling osteomyelitis.

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