Animal Models Commonly Used to Study Quorum-Sensing Inhibitors

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Abstract Multiple animal models exist for the study of biofilm infections and their inhibitors in vivo. The infection models described in this chapter range from the simple nematode-killing and amoeba-plate-killing assays, to models with more relevance to human disease like the pulmonary and cellulitis infection models in mice, the graft prosthesis, and the central venous catheter infection models in rats, and the endocarditis and osteomyelitis infection models in rabbits.

Multiple animal models exist for the study of biofilm and the effect of quorum sensing inhibitors (QSIs) in vivo. Below are a few.

1 Nematode *Caenorhabditis Elegans*

One of the simplest infectious models is in the nematode *Caenorhabditis elegans*. These 1-mm-long worms feed on bacteria from its surroundings. In a laboratory setting they feed well, for example, on a nonpathogenic *E. coli* lawn on top of an agar plate. If the *E. coli* strain is replaced by a pathogenic bacterium such as *P. aerug-*

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inosa or *S. aureus*, the worms are killed by virulence factors regulated by quorum sensing (QS). *P. aeruginosa* QS-deficient mutant, for instance, kills only 10% of the worms as compared to 100% when the worms feed on the wild-type strain. This indicates that QS is indeed important for the infectious process in *C. elegans* (Rasmussen et al. 2005a; Darby et al. 1999; Mahajan-Miklos et al. 1999; Tan et al. 1999; Sifri et al. 2003; Bae et al. 2004).

1.1 Nematode Killing Assay

Bacteria are grown overnight at 37 °C in tryptone soya (TS) broth supplemented with selective antibiotics as needed. A 1:10 dilution of the saturated culture is made in culture broth, and 10 μl of the diluted culture is spread on 3.5-cm-diameter plates containing culture broth agar supplemented with 5 μg of nalidixic acid/ml. The plates are incubated at 37 °C for 4–6 h and are then allowed to equilibrate to room temperature for 30–60 min before being seeded with worms. Next, 30–40 L4-stage nematodes are added per plate (in triplicate). The plates are incubated at 25 °C and scored for live and dead worms at least every 24 h. A worm is considered dead when it fails to respond to plate tapping or gentle touch with a platinum wire (Sifri et al. 2003).

2 Amoeba *Dicyostelium Discoideum*

The haploid social soil amoeba *Dictyostelium discoideum* has been established as a host model for several pathogens, including *P. aeruginosa*, *Cryptococcus neoformans*, *Mycobacterium* spp., and *Legionella pneumophila*. A functional RhlIR QS system has been found to be important for establishing *P. aeruginosa* infections in the amoeba, where RhlR controls the production of rhamnolipid, which lyses the amoeba (Cosson et al. 2002; Pukatzki et al. 2002; Steinert and Heumer 2005).

2.1 Plate Killing Assay

Bacteria are grown in culture broth for 16 h, pelleted by centrifugation, washed once, and resuspended in SorC (16.7 mM Na_2H/KH_2PO_4/50 μM $CaCl_2$, pH 6.0) at a final optical density of 5.5 at 600 nm. *D. discoideum* cells from midlogarithmic cultures are collected by centrifugation ($1000\times g$; 4 min), washed once with SorC, and added to the bacterial suspensions at a final concentration of 5×10^2 cells/ml suspension; 0.2 ml of this mixture is plated out on SM/5 plates and allowed to dry under a sterile flow of air. Plates are incubated for 3–5 days and examined for plaques formed by *Dictyostelium* amoebae (Pukatzki et al. 2002).

3 Mouse Pulmonary Infection Model[2,3]

A model with more relevance for humans is the pulmonary infection model in mice. The initial stages of a chronic lung infection can be mimicked by casting *P. aeruginosa* into seaweed alginate beads and surgically installing them through the trachea into the mouse lung. Under normal circumstances, the activity of the mucociliary escalator clears the lungs of foreign particulate matter such as dust and bacteria. The alginate beads partly impair the function of the escalator, and neutrophils are then recruited to the sites of infection (Pedersen et al. 1990). For a short period of time, this is reminiscent of the situation in the cystic fibrosis lung. When the mice are infected with a QS mutant, both mortality of the mice as well as horizontal spread and dissemination are significantly reduced compared to the situation with the wild type (Rumbaugh et al. 1999; Bjarnsholt et al. 2005a). If rodents are infected with a QS-deficient mutant, the immune response is faster, the polymorphonuclear leukocytes (PMNs) respond with a stronger oxidative burst, and antibodies accumulate faster in the infected lungs (Smith et al. 2002a; Wu et al. 2001). Recent research points to the fact that the wild type *P. aeruginosa* contains a QS-controlled defense system that suppresses the powerful cellular immune response by paralyzing the PMNs (Bjarnsholt et al. 2005a). For more details, see section 1 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*

4 Mouse Cellulitis Model

This model is based on the fact that staphylococci have an affinity to dextran beads with positive-charged DEAE groups throughout matrix (Cytodex microcar-

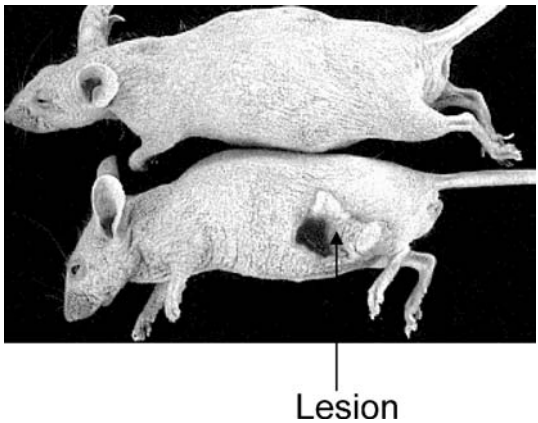

Fig. 1 Mouse cellulitis/abscess model. Mice (outbred, immunocompetent hairless male mice, strain Crl : SKH1(hrhr)Br], ($n = 10$) were injected subcutaneously with *S. aureus* and Cytodex together with the quorum-sensing inhibitor RIP (*top*) or without RIP (*bottom*). The lesion can be measured after 2 days

rier beads, GE Healthcare). When the bacteria are mixed with the beads and injected subcutaneously, a biofilm is formed, leading to a lesion or abscess that can be measured (Fig. 1). Such infections are highly dependent on the ability of the bacteria to produce toxins, and because toxin production is regulated by QS, this model is very useful in testing QSIs (Balaban et al. 1998, 2000; Gov et al. 2004).

5 Rat Graft Prosthesis Model[4,5]

The rat graft model allows for the direct quantification of bacteria on the graft itself. In the case of staphylococci, a biofilm is formed by the second day after bacterial challenge, and the graft can be removed up to 7 days after that for quantification of the bacteria on the graft. This model allows testing of compounds aimed at preventing an infection in addition to testing compounds aimed at treating an infection once a biofilm is formed.

In this model (Fig. 2), sterile collagen-sealed double velour knitted polyethylene terephthalate (Dacron) grafts are utilized as medical devices. Adult male Wistar rats ($n = 5–15$) are used, and experimental groups include control groups (no graft contamination), contaminated groups that do not receive any antibiotic or QSI prophylaxis, and treated groups that receive QSI alone or combined with other antimicrobial agents. Rats are anesthetized, the hair on the back is shaved, and the skin

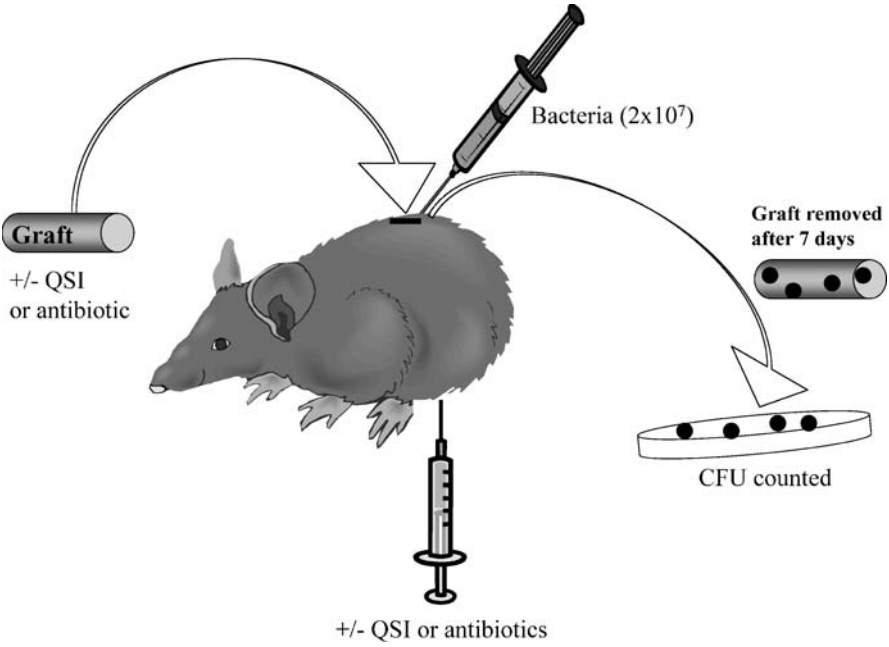

Fig. 2 Diagram of the rat graft model

is cleansed with 10% povidone-iodine solution. Subcutaneous pockets are made on each side of the median line by a 1.5-cm incision. Aseptically, 1-cm^2 sterile Dacron grafts are implanted into the pockets. Before implantation, the Dacron graft segments are impregnated (or soaked) with different concentrations of each compound. The pockets are closed by skin clips, and a physiological solution (1 ml) containing the bacteria (e. g., staphylococcal strains at a concentration of 2×10^7 CFU/ml; Balaban et al. 2005) is inoculated onto the graft surface using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals are returned to individual cages and thoroughly examined daily. All grafts are explanted at 7–10 days following implantation. The explanted grafts are placed in sterile tubes, washed in sterile saline solution, and placed in tubes containing 10 ml of phosphate-buffered saline and sonicated (Fisher Scientific 300) at 20 000 Hz for 5 min to remove adherent bacteria. Quantitation of viable bacteria is obtained by performing serial dilutions and culturing each dilution on blood agar plates. The organisms are quantitated by counting the number of colony-forming units (CFUs) per plate. In the case of staphylococci, a biofilm will have already formed by the second day. Therefore, soaking the graft in QSI before bacterial challenge allows for testing QSIs for prevention of infection, while injecting QSI intraperitoneally for 2 days postimplantation, for example, allows for testing the reagent as a therapeutic (Balaban et al. 2007).

6 Central Venous Catheter-associated Infection Rat Model[1,4,5]

This model is very relevant clinically because more than 2 500 000 central venous catheters (CVCs) are annually implanted in the United States alone (statistics obtained from the U.S. National Center for Health Statistics, 2004), and 5% of them are associated with infectious complications leading to morbidity and mortality. The CVC may become colonized by two main routes: from the skin along the outside of the catheter or via the catheter lumen (Raad 1998; Donlan et al. 2002; Atela et al. 1997). Initial colonization is followed by development of a biofilm structure, usually developing within 3 days of catheterization (Raad 1998; Hall-Stoodly et al. 2004; Costerton et al. 1999). This model was used to evaluate the efficacy of the staphylococcal QSI RIP in preventing bacterial contamination of the CVC (Cirioni et al. 2006).

As in the graft model, these studies include control groups (no CVC infection), contaminated groups that do not receive any antibiotic or QSI prophylaxis, and contaminated groups that receive QSI-treated CVC and several schemes of antibiotic lock technique therapy ($n = 12$). Animals are anesthetized. Silastic catheters are inserted into the jugular vein and advanced into the superior vena cava. The proximal portions of the catheters are tunnelled subcutaneously to exit in the midscapular space. The catheters are then flushed with a heparin solution. A rodent restraint jacket is used to protect the catheters and to allow access to them. Twenty-four hours after CVC placement, blood cultures are obtained from the catheters to verify sterility. When established (24 h after implantation), the catheters

are filled with QSI. After 30 min the rats are challenged via the CVC, such as with 1.0×10^6 CFU staphylococci in a volume of 0.1 ml sterile saline. Several animals are sacrificed 24 h after bacterial challenge to verify, by quantitative cultures, the presence of infection. At the same time, the antibiotic lock technique can be initiated for additional delivery of QSIs or antibiotics. The drugs are allowed to dwell for 1 h, and the catheters are then flushed with a heparin solution.

Quantitative peripheral blood cultures and quantitative cultures of the catheters and surrounding venous tissues can be performed as follows: On day 9 the animals are sacrificed. For quantitative peripheral blood cultures, peripheral blood is obtained by aseptic percutaneous transthoracic cardiac puncture and cultured on sheep-blood agar plates. Plates are incubated at 37 °C for 24–48 h, and the number of CFUs per plate is determined. The isolates are identified by morphological and cultural characteristics, gram stain, etc. For quantitative cultures of the catheters and surrounding venous tissues, the location of the distal tip of the CVC in the superior vena cava is confirmed visually, and the catheters and surrounding venous tissue are removed aseptically. The explanted catheters/venous tissues are placed in tubes containing 50 ml of saline solution and are sonicated (Fisher Scientific 300 at 20000 Hz for 5 min) to remove adherent bacteria. The solution is then cultured by performing serial dilutions (0.1 ml) of the bacterial suspension and by culturing each dilution and determining CFU.

7 Rabbit Endocarditis Model

Another clinically relevant model is the rabbit endocarditis model (Garrison and Friedman 1970). Bacterial endocarditis is a lethal infection that requires the administration of high levels of bactericidal antibiotics for prolonged periods of time for cure. A significant percentage of patients with endocarditis fail therapy or suffer relapse, either because resistance develops or because not all of the infection was cleared (Oramas-Shirey et al. 2001). Native valve acute endocarditis usually has an aggressive course and is often caused by *S. aureus* or group B streptococci. Alpha-hemolytic streptococci or enterococci often cause subacute endocarditis that usually has a more indolent course. Staphylococci, gram-negative bacilli, and *Candida* species often cause early prosthetic valve endocarditis, which occurs within 60 days of valve implantation. Late prosthetic valve endocarditis occurs 60 days or more after valve implantation and is often caused by alpha-hemolytic streptococci, enterococci, or staphylococci. *S. aureus* is the most common cause of endocarditis related to intravenous drug use, which commonly involves the tricuspid valve (Baddour et al. 2005).

As reported by Oramas-Shirey et al. (2001), experiments are performed on male, specific pathogen-free (SPF) New Zealand White rabbits weighing-2.0–2.5 kg ($n = 5$–15). With the use of sterile surgical technique under anesthesia, a polyethylene catheter (PE-50) is inserted into the right carotid artery and advanced

across the aortic valve into the left ventricle. The catheter is sutured in place for the duration of the study. Of note is that only data from animals with correct catheter placement upon autopsy are included. Rabbits are infected 24 h after surgery, such as with 3.5×10^6 CFU of *S. aureus* with or without QSI and/or antibiotics. Forty-eight hours after infection, control rabbits are sacrificed, and treatment with QSIs and/or antibiotics is given. Rabbits are sacrificed 8–12 h after the final dose of material in question. Aortic valve vegetations, blood, and ventricular myocardium are removed and homogenized, and quantitative bacterial counts are determined by serial dilution and expressed as CFU/g of tissue (Oramas-Shirey et al. 2001).

8 Rabbit Osteomyelitis Model

Direct observations of the surfaces of orthopedic prostheses that have failed and of bone affected by osteomyelitis with and without the presence of prosthesis have shown that the bacteria that cause these infections live in well-developed biofilms (Costerton 2005). While the following model of osteomyelitis (Brady et al. 2006; Mader 1985) requires a large organism inoculation, it produces clinical manifestations like those seen in cases of human chronic osteomyelitis, including disruption of the normal bone architecture and periosteal elevation. Also produced is the hallmark of chronic osteomyelitis, the involucrum, which is live, encasing bone that surrounds infected dead bone within a compromised soft tissue envelope (Pesanti and Lorenzo 1998; Ehrlich et al. 2004). In addition, the recalcitrance to clearance by antimicrobial agents and the host immune system that is mediated by a biofilm mode of growth is evident after 28 days of infection (Brady et al. 2006).

In this model, 8-week-old New Zealand White female rabbits ($n = 3$) are used. Rabbits are anesthetized, and an 18-gauge needle is inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. Sodium

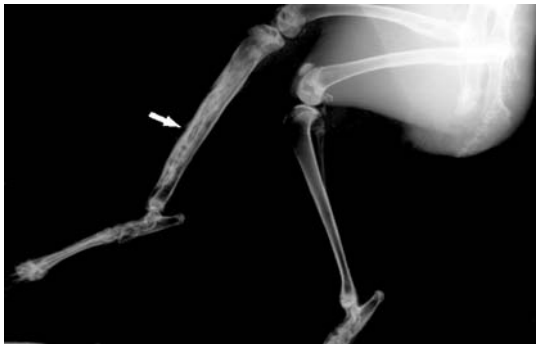

Fig. 3 Rabbit osteomyelitis model. A localized *S. aureus* osteomyelitis was surgically induced in the rabbit left lateral tibial metaphysis into the intramedullary cavity (*arrow*). Infection was allowed to progress, and the severity of osteomyelitis was determined radiographically. (Image kindly provided by Dr. Mark Shirtliff.)

morrhuate solution; 0.1 ml of *S. aureus* (10^6 CFU), for example; and sterile saline are injected sequentially. The needle is removed and the rabbits returned to their cages. The infection is allowed to progress for 42 days, with sera being drawn at days 0, 14, 28, and 42. QSI inhibitors and/or antibiotics can be administered together with the bacteria or by systemic administration at that time or after challenge. The severity of osteomyelitis can be determined radiographically at 10, 20, and 30 days following infection (Balaban et al. 2000; Fig. 3). At the conclusion of the study, rabbits are sacrificed, and both tibias are removed (one to be used as a pathogen-free control), dissected free of all soft tissue, and processed for bacterial cultures (Brady et al. 2006).

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