

The Rabbit Model of Bacterial Osteomyelitis of the Tibia

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Background of model

The treatment of acute and chronic orthopedic infections is difficult, time-consuming and expensive (Waldvogel, *et al.*, 1970; Patzakis, 1982; Cierny and Mader 1983; Cierny *et al.*, 1985; Kelly, 1986). Therefore, a variety of animal models have been used in order to test the latest antimicrobial therapies, surgical procedures, and implants for osteomyelitis. The rabbit model of osteomyelitis has been preferred by many because of the low cost, effectiveness and ease of infection initiation, low mortality rate, and close approximation to human histological and pathological patterns of this disease (Mader, 1985).

There are two current rabbit models used for the study of osteomyelitis. The Andriole model of progressive osteomyelitis was developed in 1974 and is extremely useful in the assessment of therapies associated with a complication of internal fixation (Andriole, *et al.*, 1973; 1974). In this model, *Staphylococcus aureus* is injected into the intramedullary cavity through a hole drilled through the proximal tibia. The progressive bone infection is produced through local bone trauma. This local trauma can be developed by disrupting local blood supply with the insertion of an intramedullary stainless steel nail or through a fracture of the middle third of the tibia followed by nail insertion. Both techniques were efficient in inducing progressive osteomyelitis at levels of 100% and 88% in the nail-alone rabbit group and the fracture-plus-nail rabbit group, respectively, but required a very high inoculum of *S. aureus* (10^8 cfu); Andriole *et al.*, 1973, 1974). The model produces an osteomyelitis that is classed as a Stage 1A osteomyelitis (according to the Cierny–Mader classification system) and a chronic contiguous focus osteomyelitis (according to the Waldvogel system); (Table 68.1); (Mader, 1985; Waldvogel *et al.*, 1970). Recent studies have demonstrated that a more efficient infection model uses slotted intramedullary nails instead of the solid nail that was originally used by Andriole (Melcher *et al.*, 1994). An interesting variation of the Andriole model was developed by Morrissy and Haynes (1989). This model combines tibial trauma and bacteremia for the production of acute hematogenous osteomyelitis in rabbits. In this variation to the Andriole model, the rabbit receives both a fracture injury to the proximal tibial physal

plate and a standardized bacteremia. Rabbits developed significant osteomyelitis in almost all cases. The second (and most widely used) rabbit osteomyelitis model was developed by Scheman *et al.* (1941), and was refined by Norden and Kennedy (1970). This model initially produces a contiguous-focus osteomyelitis that closely mimics acute–subacute osteomyelitis in humans. However, as the infection progresses, the development of macronecrosis is observed, consistent with chronic osteomyelitis in humans. Therefore, between 1 and 3 weeks postinfection, this model produces a diffuse 3A or 4A osteomyelitis, according to the Cierny–Mader staging system (Mader, 1985). Since the Andriole model is rarely used by investigators, we will limit our discussion to the contiguous-focus rabbit osteomyelitis model that is based upon the Norden–Kennedy rabbit model or the rabbit modification of the Fitzgerald dog model (Fitzgerald, 1983; Mader, 1985).

Table 68.1 Osteomyelitis classification systems

Waldvogel system

Hematogenous osteomyelitis
Contiguous-focus osteomyelitis
Osteomyelitis associated with vascular disease
Chronic osteomyelitis

Cierny–Mader system

Anatomic stage

Stage 1: Medullary osteomyelitis
Stage 2: Superficial osteomyelitis
Stage 3: Localized osteomyelitis
Stage 4: Diffuse osteomyelitis

Physiologic stage

A host: Normal host
B host: Systemic compromise (BS)
Local compromise (BL)
C host: Treatment worse than the disease

Animal species

New Zealand White rabbits are the species of choice and females weighing 2–3.5 kg (8–12 weeks in age) are often used because of their low level of aggressive behavior.

Preparation of animals

Once animals have arrived at your animal care facility, it is important to wait 7–10 days between arrival and initiation of infection in order to screen for those animals with infections (mainly *Pasteurella multocida*, but also *Clostridium piliformis*, or antibiotic-induced overgrowth of intestinal Gram-negatives) obtained from the commercial rabbitry source. Also, this waiting period is required for the animals to adjust to their new housing environment. In our experience, this adjustment period makes the animals more relaxed during handling.

Storage, preparation of inocula

The strain of *S. aureus* that we currently use was obtained from a patient with osteomyelitis undergoing treatment at the University of Texas Medical Branch, Galveston, TX. The strain is a methicillin-sensitive *S. aureus* (MSSA) and is stored at -70°C in defibrinated sheep blood until needed. *Pseudomonas aeruginosa*, an increasingly important pathogen in human osteomyelitis, has also been used with this rabbit model (Van Wingerden *et al.*, 1974; Dominguez *et al.*, 1975; Norden and Keleti, 1980; Norden *et al.*, 1980; Mader, 1985; Tomczak *et al.*, 1989). The infection produced by this organism is less destructive, more indolent, typified by significant bony and fibrous tissue repair, and rarely leads to extraosseous expression (Mader, 1985; Norden and Keleti, 1980). These histologic elements are somewhat different from the suppurative destructive necrosis found in the *S. aureus* osteomyelitis model. Other organisms used with this model include *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* and *Bacteroides* spp. (including *thetaiotamicro*, *melanogenicus* and *fragilis*; Mader and Adams, 1989; Mayberry-Carson *et al.*, 1990; 1992; Johansson *et al.*, 1991; Lambe *et al.*, 1991; Petri, 1991). However, in our experience at the University of Texas Medical Branch, *Staphylococcus aureus* and coagulase-negative staphylococci are the most common organisms isolated from patients with osteomyelitis (Mader *et al.*, 1997a). We will focus the description of this rabbit model using *S. aureus* as the representative infective species of bacterium.

The minimum inhibitory concentration of *S. aureus* to the test antibiotic or the antibiotic impregnated into the implant is determined using an antibiotic-tube-dilution method in cation-supplemented Mueller–Hinton broth (CSMHB); (Difco Laboratories, Detroit, MI; Ericsson and Sherns, 1971). The antibiotic of interest is serially diluted, twofold, in tubes containing 5.0 ml of CSMHB. The *S. aureus* inocula for a series of tubes is 0.1 ml of a 1.0×10^8 cfu/ml dilution in CSMHB of an overnight CSMHB culture. The minimum inhibitory concentration is the lowest concentration of antibiotic that prevents turbidity after 20 hours of incubation at 37°C . After the minimum inhibitory concentration is determined, 0.01 ml

of each clear tube is streaked on to the surface of a TSA II 5% defibrinated sheep's blood agar plate (Fisher Scientific, Pittsburgh, PA). The minimum bactericidal concentration is the lowest concentration of antibiotic that results in 10 or fewer cfu in a plated 0.010 ml sample after 24 hours of 37°C incubation.

The infective media is prepared by incubating 1 cfu of *S. aureus* overnight in CSMHB at 37°C . The bacterial concentration of the culture is adjusted to 0.5 McFarlands (10^7 cfu/ml) using a turbidimeter (Abbott Laboratories, Chicago, IL). The culture is further diluted in 0.85% saline to a final concentration of 10^7 cfu/ml.

Infection process

A contiguous focus *S. aureus* osteomyelitis is surgically induced in the left lateral tibial metaphysis of all rabbits within all study groups.

Rabbit infection

New Zealand White rabbits (Ray Nicholl's Rabbitry, Lumberton, TX), 8–12 weeks old and weighing 2.0–3.5 kg, are used for the study. After the mandatory 7 day wait following delivery to the on-site animal resources center, rabbits are anesthetized using an intramuscular injection of 45 mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 5.0–8.0 mg/kg xylazine (Rugby Laboratories, Rockville Center, NY). An 18G needle is then inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. Then 0.15 ml of 5% sodium morrhuate (Eli Lilly, Indianapolis, IN) 0.1 ml of *S. aureus* (10^7 cfu/ml) and 0.2 ml of sterile 0.85% saline are injected sequentially (Mader *et al.*, 1987; Norden, 1970; Mader and Wilson, 1983). However, it is important when working with different strains of *S. aureus* to perform preliminary experiments in order to determine the optimal bacterial inoculum. The optimum bacterial inoculum is one that induces a significant localized osteomyelitis within 2 weeks of infection without a significant increase in mortality for the 6–8 weeks of postinfection survival required for study completion.

After saline injection, the needle is removed and the animal is returned to its cage. The animal must be carefully monitored for pain for the next 24 hours. If tibial tenderness is noted (demonstrated by a resistance to bear weight on the infected leg), analgesic relief may be provided to animals by a subcutaneous dose of buprenorphine 0.01–0.05 mg/kg rabbit body weight. This dose is effective for a period of 6–12 hours. The infection is allowed to progress for 2 weeks, at which time the severity of osteomyelitis is determined radiographically (Table 68.2) and the rabbits that demonstrate a 3+ to 4+ radiographic severity are divided into treatment groups.

Table 68.2 Criteria for grading the radiographic severity of *Staphylococcus aureus* osteomyelitis in rabbits; percentages of disrupted bone are visually estimated from the radiographs

Severity rating	Radiographic characteristics
0	Normal
1+	Elevation or disruption of periosteum, or both; soft tissue swelling
2+	< 10% disruption of normal bone architecture
3+	10–40% disruption of normal bone architecture
4+	> 40% disruption of normal bone architecture

Antimicrobial therapy

Treatment groups when testing new antibiotics

At the end of 2 weeks postinfection, the rabbits with *S. aureus* contiguous-focus proximal tibial osteomyelitis are separated into study groups with each group being sacrificed at 8 weeks postinfection. Group One; this group of rabbits is infected but left untreated for the duration of the study. Group Two; rabbits in this group are treated for 4 weeks with a standard antibiotic used in the treatment of human *S. aureus* osteomyelitis (such as parenteral vancomycin or nafcillin). Group Three; rabbits are treated for 4 weeks with the experimental antibiotic. The dose levels of the standard and experimental antibiotics should be chosen so they will achieve serum levels in the rabbits that are comparable to optimum serum levels suggested for humans. If desired, one may include other groups in which rabbits are treated for 4 weeks at a different dose level. Each group should consist of 15–20 rabbits in order to attain statistical significance of results and compensate for the estimated 5–10% of rabbits that die before study completion.

At the end of 28 days (a total of 42 days postinfection) of therapy, radiographs are repeated and compared to the pre-treatment radiographs to evaluate the extent of osteomyelitis, the effect of therapy, and the bone growth. In order to accurately assess the effectiveness of the treatment modalities, the rabbits remain untreated for 2 more weeks following the end of antibiotic treatment (a total of 42 days postinfection). Animals are radiographed and sacrificed at this time and all tibias are harvested for bone *S. aureus* concentration determination. The rabbits are monitored daily for stool character, weight loss, caloric intake, tibial tenderness, and overall general health.

Treatment regimen when testing new implant material

In-vitro antibiotic elution from implant material

If the *in-vitro* elution rate of the study antibiotic from the implant material has not previously been done, an *in-vitro*

determination of elution rates should be performed (Mader *et al.*, 1997b). An antibiotic-loaded bead (3–6 mm diameter) constructed of the implant material of interest is submerged in 1 ml of phosphate-buffered saline (pH 7.4) for 24 hours at 37°C. At 24-hour intervals, the phosphate buffer is drawn off and the antibiotic-loaded beads are then resubmerged in fresh buffer. This procedure is repeated until the bead is fully dissolved or the eluted antibiotic levels are undetectable. All samples should be kept frozen at –70°C until antibiotic concentrations are determined by the disk diffusion method (see below). Obviously, if implants are to be constructed into shapes or sizes different than beads, appropriately adjust the amount of PBS added.

Treatment groups when testing novel antibiotic-impregnated implants

At the end of 2 weeks postinfection, the rabbits with contiguous-focus proximal tibial osteomyelitis are separated into study groups and each group is sacrificed at 8 weeks postinfection. We list here some suggested groups that researchers may wish to include in a study that evaluates novel antibiotic-impregnated implants.

- Group One; this group of rabbits is infected but left untreated for the 8-week duration of the study.
- Group Two; rabbits in this group undergo debridement surgery 2 weeks postinfection.
- Group Three; rabbits undergo debridement surgery (2 weeks after infection) and 4 weeks of appropriate antibiotic administration (subcutaneous, intramuscular, intravenous, or oral).
- Group Four; rabbits have only 4 weeks of appropriate antibiotic administration starting 2 weeks postinfection.
- Group Five; rabbits have the experimental implant material (without antibiotic impregnation) implanted into the dead space created by the debridement surgery (performed 2 weeks after infection).
- Group Six; rabbits in this group have the experimental implant material (without antibiotic impregnation) implanted into the debrided region and appropriate antibiotic administration at the correct dosage for 28 days following debridement surgery.
- Group Seven; rabbits in this group have the experimental material (impregnated with tobramycin, vancomycin, or other standard implanted antibiotic) implanted into the dead space left after the debridement surgery.
- Group Eight; rabbits in this group have polymethylmethacrylate (PMMA) beads (impregnated with the same standard implanted antibiotic as above) implanted into the dead space after debridement surgery.

The choice of appropriate subcutaneous, intramuscular, intravenous, or oral antibiotic will depend upon the type of infecting organism that will be tested (see Table 68.3 for some examples) and dosages of these antibiotics are chosen

Table 68.3 Type of bacterial species used for disk diffusion seed organism when testing specific antibiotics

Test antibiotic	Bacterial species for disk diffusion
Cefamanadole	<i>Bacillus subtilis</i> (Difco)
Cephazolin	<i>Bacillus subtilis</i> (Difco)
Cephalothin	<i>Bacillus subtilis</i> (Difco)
Ciprofloxacin	<i>Bacillus subtilis</i> (Difco)
Ofloxacin	<i>Bacillus subtilis</i> (Difco)
Rifampicin	<i>Bacillus subtilis</i> (Difco)
Ticarcillin	<i>Bacillus subtilis</i> (Difco)
Tobramycin	<i>Bacillus subtilis</i> (Difco)
Vancomycin	<i>Bacillus subtilis</i> (Difco)
Azithromycin	<i>Micrococcus luteus</i> (ATCC 9341)
Clarithromycin	<i>Micrococcus luteus</i> (ATCC 9341)
Clindamycin	<i>Micrococcus luteus</i> (ATCC 9341)
Daptomycin	<i>Micrococcus luteus</i> (ATCC 9341)
Nafcillin	<i>Micrococcus luteus</i> (ATCC 9341)
Tosufloxacin	<i>Escherichia coli</i>
Cefdinir	<i>Providencia stuartii</i> (ATCC 43665)

to produce serum levels that are consistent with those suggested for human. However, for all implants, we generally use vancomycin as the impregnating antibiotic, but other implanted antibiotics, including tobramycin, clindamycin, and gentamicin (in Europe), have been successfully used. As was true with group sizes when testing new antibiotics, each group should consist of 15–20 rabbits in order to attain statistical significance of results and compensate for the estimated 5–10% of rabbits that die before study completion.

The implant material should not be packed within the dead space to fill the entire debrided area, but should be placed within the defect as beads of less than 6mm diameter, for a number of reasons. First, by packing the entire dead space with the implant material, the immune system and blood supply become locally compromised, leading to hindrance of infection resolution. Second, by having a single, solid implant, the amount of surface area is much lower than that derived from beads (Gristina, 1994). This lower surface area prevents effective resorption, dissolution, or degradation of a degradable implant. Also, in antibiotic impregnated material, a lower surface area reduces the effectiveness of antibiotic elution from the material, leaving a largely unused residual amount of antibiotic in the center of the single, solid implant (Calhoun and Mader, 1997; Mader *et al.*, 1997). Lastly, by implanting a large amount of material that has a slow dissolving rate and a low antibiotic elution profile, one provides a long-term substrate for the attachment and eventual colonization of pathogenic organisms (Gristina, 1994).

A 4-week therapy period is required for each group (except Group One). At the end of 4 weeks of therapy, radiography is repeated and compared to the pretreatment radiographs to evaluate the extent of osteomyelitis, the effect of therapy and the bone growth. The rabbits are sacrificed after the radiographic study. All groups should

have histologic studies performed on the tibiae (three per group) at the end of the 8-week study. The remaining tibiae from each group are used for quantitative *S. aureus* concentration determination.

Treatment regimen when testing a new osteomyelitis detection method

Rabbits should initially be divided into two groups before the infection procedure. Group one rabbits are infected as above in the left leg. However, when testing a new detection method, it is prudent to also insert an 18G needle, percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. This is followed by sequential injections of 0.15 ml of 5% sodium morrhuate (Eli Lilly, Indianapolis, IN) and 0.3 ml of sterile 0.85% saline. This internal control is necessary since radiographic, magnetic resonance imaging, and some nuclear studies (unpublished data) demonstrate that the sclerosing agent of sodium morrhuate alone can yield images that are similar to those produced by infection at early stages after inoculation (Volk *et al.*, 1994). These Group One rabbits should be imaged before infection and at 3, 7, 14, 21, and 28 days following infection, using a gold-standard method of osteomyelitis detection (such as radiographs or three-phase bone scans). Group Two rabbits should have identical procedures performed on them, except that detection uses the new osteomyelitis detection method. All rabbits upon sacrifice or death should have histological and culture results that prove the existence of *S. aureus* osteomyelitis.

Details of surgery

Required personnel

Surgery requires three participants. A surgeon and an assistant are required to perform the actual surgery and both are sterile from the waist to the neck, including arms and hands. A 'third out' is also required; she is responsible for animal preparation, animal and material transport, preparing the surgical area, casting, and all other actions that would compromise the sterility of the surgical area and attendees.

Materials for surgery

The materials that must be sterilized and remain in the sterile surgical field are placed on a surgical tray, double-wrapped with cotton sheets, and sterilized (121°C, 15Atm, 30 minutes). This allows the sheet to be opened without compromising the sterility of any of the instruments on the tray. These surgical tray instruments are

used to perform the surgery and include: two disposable aluminum cups, gauze pads, two scalpel handles, two surgical blades (no. 10), two short-straight needle holders, two short-curved needle holders, two surgical straight scissors, four towel clips, two spatulas, one forceps, one tissue forceps, one bone saw blade, and four surgical towels. Other tools needed for the surgery but not placed on the surgical tray include: two ready-made syringes of the ketamine/xylazine cocktail (see above), scissors for leg preparation, gauze pads (non-sterile), 70% isopropyl alcohol swabs, surgical gowns, surgical gloves, surgical hats, 4-0 chromic gut sutures, 4-0 prolene sutures, implant material, powdered antibiotic, extra 1 ml and 3 ml syringes, and a weighing scale (0.1–20.0 g). All surgical materials can be obtained from Fisher Scientific (Pittsburgh, PA) except sutures, which may be obtained from Ethicon (Somerville, NJ).

Surgical preparation

It is highly recommended that personnel performing the surgeries be well-trained by individuals proficient in sterile technique surgery. The entire surgical area is cleaned and sterilized using a 10% bleach solution. Immediately before the animal is brought into the surgical area, the surgical tray is placed on one end of the table. It is then opened so that none of the sterile field inside the sheet is broken, and the inside of the sheet is facing up. This provides a sterile area for the rabbit to be placed upon. The bone saw is also wiped, cleaned and sterilized with a 10% bleach solution by the third out. A final pass with bleach is made starting at the cutting end of the saw and working down towards the cable, never moving back toward the tip of the saw. The saw is then placed in its designated area within the sterile field.

Animal preparation

The animal is first anesthetized using an intramuscular injection consisting of ketamine (45 mg/kg) and xylazine (6.0–8.0 mg/kg). After the animal is properly anesthetized, it is brought into the preparation area. All the hair from the infected leg should be removed, using scissors to cut the hair, getting as close to the skin as possible. The entire leg is then washed with liberal amounts of alcohol, followed by a thorough washing and wiping of the area with clinidine solution. This should remove any of the normal skin flora that could enter the surgical site during the procedure. The cleaned leg should now be considered a sterile site, and should not come into contact with other objects, including the rabbit itself. After the rabbit is prepared for surgery, it is brought in and laid down on to the sterile sheet. During the transit, care is taken that the prepared leg does not come into contact with any contaminants.

Surgeon preparation

The surgeon opens the package containing the surgical gown and the package containing the surgical gloves in a designated area of the surgical suite. First, the surgeon puts on a surgical hat and mask, then scrubs in using an iodine scrub pack, following standard surgical procedure. This consists of making sure that the hands remain at an elevation above the rest of the forearm at all times. The hands receive the most attention, scrubbing each surface area of the hand at least ten times. The forearm is also scrubbed vigorously to remove any microorganisms. When rinsing the hands, the hands still remain above the elbows so that the water rinses from the hands down, to keep any microbes from less scrubbed areas from landing on the hands. Maintaining the sterility of the hands, the surgeon enters the surgical suite and dons the surgical gown and gloves. The sterile field on the surgeon is considered to be from the waist up to the neck including the lengths of both arms.

Surgical procedure

All sterile materials for surgery are derived from the surgical tray. Once the rabbit is placed on the sterile sheet, the surgical towels are draped over and attached to the animal (using towel clips) to expose the infected leg. Then, using the number 10 scalpel, an incision is made just through the dermal layer along the medial aspect of the tibia, approximately 2 cm below the tibial metaphysis, and about 1 cm in length. After a small incision has been made, surgical scissors are used to cut the skin through the cutaneous layer, but not through the underlying fascia. The total length of the incision is about 4 cm. The plane of dissection can be seen and felt grossly by locating the lateral edge of the tibia and the incision should follow this edge. The exact line of dissection is a white line separating the two muscles, *musculus tibialis cranialis* and *musculus extensor digiti II* (Figure 68.1). The next incision is made through the muscle fascia. A very slight pressure is applied to the scalpel so that the blade does not injure the underlying muscle. The next incision is through the aforementioned white line in order to separate the two muscles (Figure 68.2). If at any time, bleeding ensues, then sterile gauze is placed on the site, and pressure is applied until the bleeding stops.

A gauze dissection is then performed to separate the muscles and expose the tibia. A piece of sterile gauze from the surgery tray is placed on each muscle at the zone of incision, and the two muscle groups are pulled away from each other (Figure 68.3 and 68.4). This method allows the muscles to be moved while sustaining little or no damage and leaving none of the underlying fascia attached to the bone. At this point, the area of bone window to be removed is determined and this is usually done by examining the radiograph of the animal. The area that contains the



Figure 68.1 After a small incision has been made, surgical scissors are used to cut the skin through the cutaneous layer, but not through the underlying fascia. The total length of the incision is about 4 cm. The plane of dissection can be seen and felt grossly by locating the lateral edge of the tibia; the incision should follow this edge. The exact line of dissection is a white line separating the two muscles, *musculus tibialis cranialis* and *musculus extensor digiti II*.

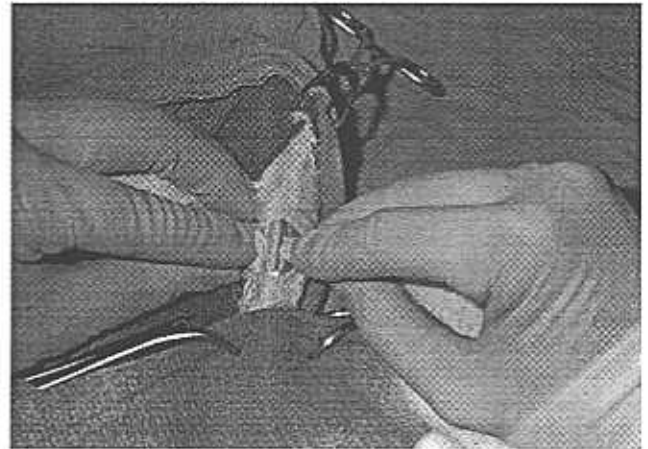


Figure 68.3 A gauze dissection is then performed to separate the *musculus tibialis cranialis* and *musculus extensor digiti II* and expose the tibia. A piece of sterile gauze from the surgery tray is placed on each muscle at the zone of incision, and the two muscle groups are pulled away from each other.

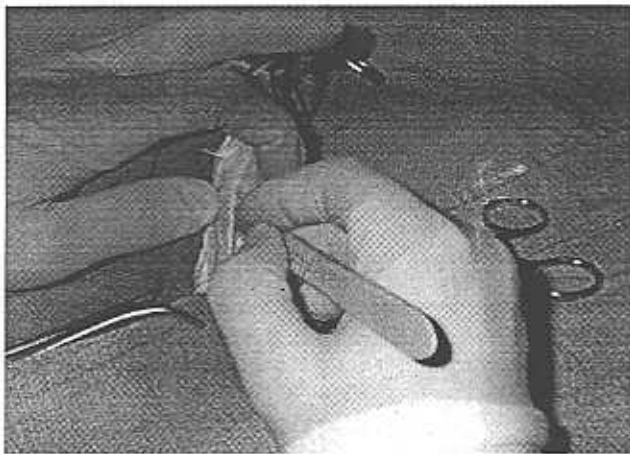


Figure 68.2 The next incision is made through the muscle fascia. A very slight pressure is applied to the scalpel so that the blade does not injure the underlying muscle. The next incision is through the aforementioned white line in order to separate the two muscles.

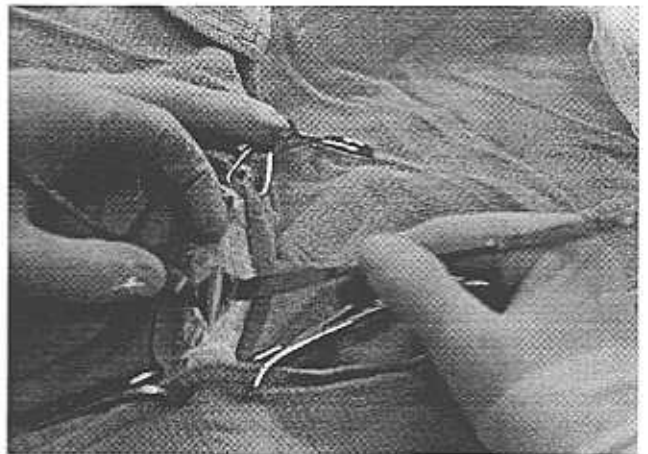


Figure 68.4 Using two spatulas, the surgeon displays the infected left tibia following gauze dissection.

greatest concentration of infection should be the initial location of debridement.

Debridement starts with the removal of a bone flap approximately 0.5 cm wide by 2.0 cm long using a Stryker 40 k command sag saw, with the angle saw blade (Stryker Corporation, Kalamazoo, MI). The bone flap is then removed. The infected area is easily detected as a yellow purulent substance, as opposed to the deep red color of normal marrow. Using the narrow end of the spatula, the visible infection is removed. However, there is usually more infection spread throughout the bone. The amount of debrided bone should be kept to a minimum, as large areas

of removal will decrease bony stability. However, it is imperative to remove all sequestra and discolored bone marrow (Figure 68.5).

Surgical excision of the bone is carried down to uniform haversian or cancellous bleeding, termed the paprika sign. While this technique will not completely sterilize the bone, it will remove the bone sequestra and the majority of the infecting pathogen, allowing antibiotics, implants, and/or the rabbit immune system to suppress the infection.

The implant should now be placed into the dead space created by the debridement. The implant material is prepared in the sterile field, so that it will not pose any threat by becoming contaminated and infecting the host. The experimental implant material is prepared by placing a known amount of the material into one of the aluminum cups on the surgery tray. Then, saline (and antibiotic if

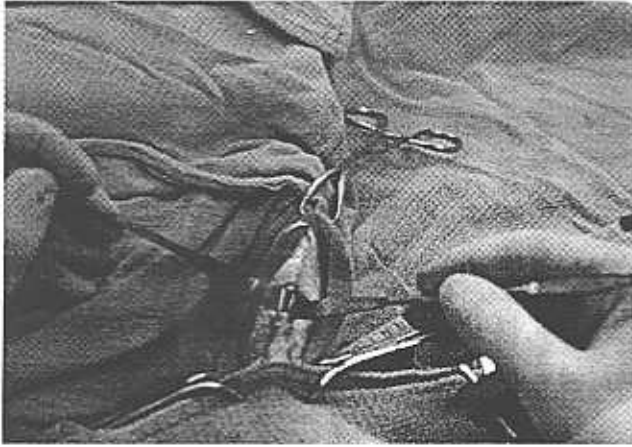


Figure 68.5 The area that contains the greatest concentration of infection should be the initial location of debridement. Debridement starts with the removal of a bone flap approximately 0.5 cm wide by 2.0 cm long using a Stryker 40k command sag saw. The bone flap is then removed. The amount of debrided bone should be kept to a minimum, as large areas of removal will decrease bony stability. However, it is imperative to remove all sequestra and discolored bone marrow.

necessary) are added to the cup, and the substances are mixed together until the desired consistency is obtained. It will often require preliminary experiments to determine the amount of antibiotic and saline to add to the experimental implant in order to obtain a correct consistency. PMMA beads are prepared in a similar manner (see manufacturer's directions, Howmedica, Rutherford, NJ). As soon as a homogenous PMMA consistency is reached, small beads are rolled out by hand. This must be done quickly, as the PMMA reacts quickly, and becomes solid within a few minutes. While it is up to the discretion of the researcher as to what shape the implant will take, we have had numerous successes with multiple beads 3–6 mm diameter) implanted into the dead space left by debridement surgery.

After the implant is in place, the surgical site is closed. It is suggested that the bone flap is left out and not secured with bone wax since this material seems to act as an attachment material for *S. aureus* to colonize and form a biofilm. The muscles that were separated at the beginning of the surgery are reattached with Ethicon chronic gut sutures, size 4–0 (tapered needle). The suturing line should be along the same line as the original ligament that was dissected. The sutures are tied so that the muscles are touching, but are not pulled together so tight that there is a bunched group of muscle at the dissection line. This procedure is repeated down the dissection line every 0.5 cm. The end result should be muscles that are attached along the same line as the original dissection. The outer cutaneous skin is sutured in the same manner, except that Ethicon Prolene 4–0 sutures with a cutting needle are used. If, at any time during the procedure, bleeding starts to occur, the procedure is halted and sterile gauze is placed over the bleeding site with adequate pressure until the bleeding stops. After the bleeding stops, the procedure resumes.

Postoperative procedure

After the surgical incision has been closed, and no bleeding is visible, the animal is removed from the surgical suite and taken back into the preparation area. The debridement of the tibia usually causes some bone instability so a fiberglass cast can be used to provide external fixation. Once inside the preparation room, the leg is thoroughly washed with saline. Four-inch gauze is then wrapped around the leg, starting at the proximal portion of the leg and working down towards the foot. Next, a cotton layer (1" Sof-Rol cast padding, Fisher Scientific, Pittsburgh, PA) is wrapped around the leg in the same fashion as the gauze. Then 3M Scotchcast Plus fiberglass casting, size 3" (3M Healthcare, St Paul, MN) is applied, starting from the proximal end of the leg and progressing down towards the distal end of the foot. Care must be taken to ensure proper cast fit. A cast too loose, will not serve its purpose, and a cast too tight can cause a number of problems, including tissue ischemia and inflammation. Also, at the proximal end of the cast, there needs to be ample amounts of Sof-Rol protruding from the top, so that it can provide a barrier against the cast rubbing the leg and side of the animal to a point of infection. After the casting has been applied, it is shaped to the curvature of the animal's leg. The shape of the casting should correspond to the position of the rabbit sitting at rest, allowing the rabbit to rest comfortably once it recovers from surgery (Figure 68.6). Before the rabbit is returned to its cage, it is given a postsurgical analgesic, Buprenex, at 0.1 mg/kg administered subcutaneously. Another 0.1 mg dose is given 24 hours postoperatively.

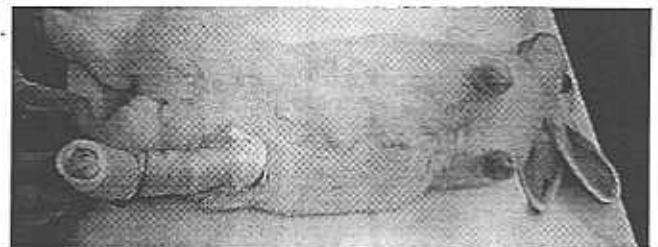


Figure 68.6 Following Soft-Rol application, 3M Scotchcast Plus fiberglass casting (size 3") is applied, starting from the proximal end of the leg, and progressing down towards the distal end of the foot. Care must be taken to ensure proper cast fit. A cast too loose, will not serve its purpose, and a cast too tight can cause a number of problems, including tissue ischemia and inflammation. Also, at the proximal end of the cast, there must be an ample amount of Sof-Rol protruding from the top, so that it can provide a barrier against the cast rubbing the leg and side of the animal to a point of infection. After the casting has been applied, it is shaped to the curvature of the animal's leg. The shape of the casting should correspond to the position of the rabbit sitting at rest, allowing the rabbit to rest comfortably once it recovers from surgery.

Key parameters to monitor response to treatment

Simultaneous level measurements in serum and bone

A group of uninfected animals and a group of 3-week-infected animals are administered a single dose of a standard antibiotic used in the treatment of osteomyelitis. To another group of uninfected animals and a group of 3-week-infected animals, the experimental antibiotic is administered by a single dose. The antibiotic dose amounts of each antibiotic are given at a level that provides serum concentrations that correspond to desired human serum levels in treating osteomyelitis. At 1, 3, 6, 12, and 24 hours following administration of either antibiotic animals for each group at each timepoint have serum drawn, and the right and left tibias are harvested (following sacrifice) for antibiotic concentration determination (see below). In order to obtain serum samples, the animal is first anesthetized with the normal dose of 45 mg/kg ketamine plus 5.0–8.0 mg/kg xylazine. The animal is then laid in a lateral position with the ear in an inferior position to the rabbit body, until the major ear vein becomes prominent. Blood is slowly drawn (0.5 ml) from the major ear vein with a 25 g needle and placed in a sterile microcentrifuge tube in order to allow for coagulation at room temperature. It is very important to note if the test antibiotic is light-sensitive because these require storage and testing in minimal ambient light.

However, in studies testing the efficacy of antibiotic-impregnated implants, a systemic and intramedullary serum samples must be obtained. In this case, a group of uninfected animals and a group of 2-week-infected animals undergo debridement surgery followed by standard antibiotic-impregnated experimental material implantation on the same day. Another group of uninfected animals and a group of 2-week-infected animals undergo debridement surgery followed by standard antibiotic-impregnated polymethylmethacrylate (PMMA; Howmedica, Rutherford, NJ) bead implantation on the same day. Serum and bone biopsy samples are obtained at this time then again at 1, 2, 3 and 4 weeks following debridement surgery and material implantation. Systemic serum samples are obtained from anesthetized animals as described above. However, the procedure for obtaining the intramedullary serum sample is somewhat more complex. A small window (2.0 × 2.0 cm) is cut into the cast about 3–4 cm distally from the surgical site. The underlying cotton wrap is then removed to provide clear access to the leg. An 18G needle is inserted into the bone allowing for approximately 0.5 ml of blood to be drawn from the intramedullary space. The inserted needle may need to be removed and a fresh one inserted through the newly created channel, as the first needle may have a bone blockage, preventing the blood from being drawn. This procedure may be repeated on the non-casted, uninfected

right tibia. All blood samples are allowed to coagulate at room temperature, then the serum is drawn off and stored at –70°C until the antibiotic concentrations in these samples can be determined (see below).

Drug assays in serum and bone

An agar-disc-diffusion bioassay is used to measure antibiotic concentrations in both serum and bone eluates. Seeded agar test organisms are usually chosen in conjunction with the pharmaceutical company. Standards of antibiotic are created by serially diluting a 1000 µl/ml stock solution in 100% normal rabbit serum (Sigma Chemical Co., St Louis, MO) and storing these dilutions at –70°C. Volumes of 20 µl of serum standards or serum samples are placed on blank concentration discs (4 Difco Laboratories, Detroit, MI), placed on the seeded agar plates, and incubated at 37°C overnight. The diameters of the zones of inhibition of test bacteria growth are measured for both samples and standards. Unknown concentrations are determined from semi-log standard curves plotted from standards. Also, blood is drawn from each rabbit prior to the administration of antibiotic. If inhibitors are detected the animal should not be used in the study.

Infected and uninfected bone is prepared for assay by dissecting them free of all soft tissue, breaking them into small pieces, and removing the marrow. The small bone pieces and the marrow are then separately weighed and suspended in 50% 0.1 M sodium phosphate buffer, pH 7.5, and 50% normal rabbit serum at a rate of 1 ml of the buffer-serum solution is per 0.5 g bone/marrow. The bone and marrow samples are then agitated in an Erlenmeyer flask for 1 hour at 4°C with a magnetic stirring bar. Antibiotic levels in the supernatant fluid are assayed by the previously described disc-diffusion methods. Standard solutions of the drugs are prepared by adding normal uninfected bone to the buffer-serum solution containing known amounts of antibiotic.

Radiographs

Radiographs are taken at infection surgery, at initiation of therapy 2 weeks postinfection, and at therapy termination (6 weeks postinfection). Radiographs may also be taken just before sacrifice (eight weeks post-infection). Radiographs should be comparatively scored (see Table 68.2) by double blind evaluation.

Determination of bacterial concentration per gram of bone

Quantitative counts of *S. aureus* cfu per gram of tibial bone should be determined for all study groups. After animals are sacrificed, the right and left tibias are stripped free of all

soft tissue, broken into small fragments, and all adhering bone marrow is removed and separated. Bone and marrow samples are separately weighed, physiologic (0.85%) saline is added in a 3:1 ratio (3 ml saline/g of bone or marrow) and the suspension is vortexed for 5 minutes. Seven 10-fold dilutions of each of the saline-bone or saline-marrow suspension are prepared with sterile 0.85% NaCl solution; 20 μ l samples of each of the seven dilutions are spotted in triplicate on to 5% TSA II defibrinated sheep's blood agar plates (Fischer Scientific, Pittsburgh, PA) and incubated at 37°C for 24 hours. Be sure to give the 20 minutes samples enough time at room temperature to absorb into the agar before moving them to the incubator. Cfus are then counted for each tibia sample. The mean log of the cfu for the dilutions and the mean *S. aureus* concentration for each treatment group can then be calculated. It is always important to check isolated colonies with a Becton Dickson Staphyloside test (Becton Dickson & Co., Cockeysville, MA) or other related coagulase test to accurately identify *S. aureus* colonies. If contamination of the plate occurs, streak the most concentrated dilution on to mannitol salt agar plates (Fischer Scientific, Pittsburgh, PA), a media that is selective for *S. aureus* colonies.

Pitfalls (advantages/disadvantages) of the model

While this rabbit model has proved to be successful in the study of osteomyelitis, it does have a few limitations. First, the model requires a very high inoculum of bacteria (10^5 – 10^6 cfu for *S. aureus* and up to 10^8 cfu for *P. aeruginosa*) to produce an adequate experimental osteomyelitis. This is unlike the human condition, in which even a very small bacterial inoculum can produce osteomyelitis. Second, in previous studies while testing a new antibiotic in rabbits with experimental osteomyelitis, rabbits were treated with the antimicrobial without any surgical intervention. In the clinical setting, however, osteomyelitis is best resolved by the combination of surgical procedures and antibiotic management. Infected patients usually undergo debridement surgery in order to remove bony sequestra and areas of aseptic necrosis. Therefore, this procedure should be considered when testing the *in-vivo* efficacy of a new antimicrobial agent in rabbits. Third, sodium morrhuate produces a small focus of fibrosis at the inoculation site, often mimicking the histological and imaging characteristics of the osteomyelitis infection pattern. Therefore, when testing a new imaging procedure, it is extremely important to employ negative control rabbits that have sodium morrhuate but not bacteria injected into their tibias. Fourth, the infection has been shown to clear with time, especially when osteomyelitis is induced by *P. aeruginosa*. Fifth, because of the rabbit's small size, many surgical procedures are either impossible or require extreme

precision. Some of these procedures include saucerization, local muscle flaps, and free flaps. Lastly, the use of long-term, high-dose antibiotic regimens in rabbits results in significant morbidity and mortality due to the heightened sensitivity of rabbits to some of the toxic effects of certain antibiotics. In particular, nafcillin administration often leads to excessive diarrhea, weight loss, and dehydration in rabbits treated for more than 7 days. If left untreated, mortality rates can reach as high as 50–60% (unpublished data). However, treatment with a daily subcutaneous injection of 50ml of lactate Ringer's solution and a special diet including cabbage and alfalfa reduces the mortality rates to acceptable levels (5–10%).

While it is obvious that this model is imperfect, the rabbit model of osteomyelitis has been used for a number of studies because of its significant advantages. First, it has lent support to many clinical observations and has successfully been used to assess pathophysiologic and therapeutic variables. Next, rabbit osteomyelitis has the advantage of demonstrating very similar pathologic characteristics and histologic properties to human osteomyelitis. This model is also reliable, inexpensive, and the animals are large enough to allow some surgical manipulation. Lastly, this model also allows for the specific and controlled study of osteomyelitis without the interference of multiple variables. Although a perfect model has yet to be invented, the rabbit model of osteomyelitis has definite advantages when human studies are inappropriate or unavailable.

Contributions of the model to infectious disease therapy

Besides being used for a variety of molecular investigations of infectious agents (Costeron *et al.*, 1987; Mayberry-Carson *et al.*, 1986; Gillaspay *et al.*, 1987), this rabbit model of osteomyelitis has been used to determine the effectiveness of experimental antibiotics and the levels attained by these antibiotics in the tissue and serum of infected and non-infected animals (Van Wingerden *et al.*, 1974; Norden, 1975, 1978; Mader *et al.*, 1978; Mader and Wilson, 1983; Norden and Shaffer, 1983; Norden *et al.*, 1986a,b; Mader and Adams, 1989; Mayberry-Carson *et al.*, 1990; Norden and Budinsky, 1990). Other studies that have utilized this model include histologic studies (Crane *et al.*, 1977), hyperbaric oxygen evaluation (Mader *et al.*, 1978, 1980; Esterhai *et al.*, 1986), determination of tissue oxygen tensions (Mader *et al.*, 1978; Aro *et al.*, 1984), blood flow studies (Mader *et al.*, 1980), new bone implants (either alone or impregnated with antibiotics) (Tomeczak *et al.*, 1989; Thomas *et al.*, 1989; Laky *et al.*, 1983; Lambe *et al.*, 1991; Wei *et al.*, 1991; Mayberry-Carson *et al.*, 1992; Melcher *et al.*, 1994; Tsouvakas *et al.*, 1996; Heard *et al.*, 1997), and new osteomyelitis detection methods (Risky *et al.*, 1977; Alzraki *et al.*, 1984; Hartshorne *et al.*, 1985; Abiri *et al.*, 1992; Volk *et al.*, 1994).

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