A Non-traumatic Staphylococcus aureus Osteomyelitis Model in Pigs

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Abstract. Background: The propensity for bacterial localization within bones of juvenile pigs is similar to the situation in humans, where haematogenously based osteomyelitis most commonly occurs in infants and children. In both pigs and humans, Staphylococcus aureus is a dominant cause of pyaemic lesions including osteomyelitis. The aim of the present study was to evaluate the pig as a model for the development of osteomyelitis following haematogenous spread of S. aureus. Materials and Methods: Twelve animals were challenged intravenously once or twice with $1 \times 10^8$ bacteria/kg body weight and euthanased consecutively from 6 h to 48 h after challenge. Following euthanasia, tissues were sampled from the lungs and bones for histology and immunohistochemical staining of vessels, different inflammatory cells, apoptotic cells, and S. aureus. Results: Disseminated microabscesses developed within the lungs by 6 h but had disappeared at 48 h. Within the metaphyseal area of bones, microabscesses developed after 12 h and progressed until 48 h after challenge. Within bones, lesions were localized in separate foci from where the infection progressed towards the growth plate, which was in some cases bypassed due to bacterial spread through transphyseal vascular channels. Often, bone lesions resulted in trabecular osteitis, in which apoptotic cells were sometimes present. Conclusion: The model revealed a pattern of development and presence of lesions similar to the frequently occurring osteomyelitic lesions, especially in prepubertal children following haematogenous spread of S. aureus. Therefore, this model can be reliably applied in studies of this disease with respect to pathophysiology, pathomorphology, impact of strain virulence, and therapy.

The incidence of human osteomyelitis due to Staphylococcus aureus has increased dramatically in recent years (1). Infections with antibiotic-resistant S. aureus have put further focus on the risk of a negative outcome of therapy leading to pathological fractures and other disablements, which have also increased in recent years (2). Osteomyelitis is frequent in pigs, and in Denmark more than 100,000 pigs are annually diagnosed at slaughter with pyaemic lesions primarily located within the lungs and bones (Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration, 2008, unpublished data). The propensity for localization within bones of juvenile pigs is similar to the situation in humans, where haematogenously based osteomyelitis most commonly occurs in infants and children (3, 4). In both pigs and humans, S. aureus is a dominant cause of pyaemic lesions including osteomyelitis (4-6).

A number of animal models of osteomyelitis have been developed for investigating e.g. pathogenesis, diagnosis, and treatment of bone infections due to S. aureus. In these models, several animal species such as rabbit, rat, guinea pig, chicken, dog, goat, and sheep have been used (7). It seems that the rabbit is often preferred since it is more prone to infection than other animal species, easily housed, and possesses a suitable size compared to mice and rats. However, a major drawback using rabbits is their pseudoruminant gastrointestinal system, which precludes testing of many antibiotics (8). In the different animal models of osteomyelitis, two main routes for bacterial inoculation have been applied, namely the haematogenous route and the traumatic route. The haematogenous route can be further divided into systemic spread to bones following inoculation of peripheral veins (9) and inoculation of nutrient arteries to bones (10), respectively. In the traumatic models, inoculation of bacteria is carried out through a hole drilled in the bone (11). The traumatic

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route does not meet the requirements of a discriminative animal model (12) because by far, most cases of osteomyelitis in humans are established following haematogenous spread of bacteria (3, 4).

In pigs, only two experimental osteomyelitis models have been reported (13, 14). Both models were based on traumatic techniques used for the inoculation of *S. aureus* into the medullar cavity of the femoral (13) and mandibular bone (14), respectively. Moreover, as well as creating artificial necrosis prior to the bacterial inoculation, a sclerosing material (sodium morrhuate) was also applied together with foreign body material (bone wax or polymethylmethacrylat) in one of the models (14). Although osteomyelitis was successfully developed in both models, severe problems with bacterial contamination were apparent in both porcine models (13, 14).

The aim of the present study was to evaluate the pig as a model for the development of osteomyelitis following haematogenous spread of *S. aureus*. Apart from being a discriminative animal model compared with previous porcine models of osteomyelitis, a model in pigs would also be advantageous compared to models in other animal species in treatment trials for antibiotic therapy due to the similarity of human and porcine physiology, metabolic rate, and development of organs (15, 16). Moreover, a porcine model will also meet the need of non-rodent models of diseases, which are a prerequisite for the biopharmaceutical industry as an auxiliary tool for testing candidate drugs prior to registration. A final advantage arises from the fact, that *S. aureus* is a frequent cause of spontaneous porcine pyaemic lesions including osteomyelitis (5, 6).

**Materials and Methods**

**Animals and housing.** A total of 16 clinically healthy female Yorkshire-Landrace crossbreed 8-week-old pigs with a body weight (BW) of 20-25 kg were used. The animals originated from a commercial specific pathogen-free herd (17). At arrival, the animals were placed in two pens and allowed to acclimatize for 7 days before entering the trial. The animals were fed a commercial pig diet (Svine Erantis Brogaarden ApS, Lyng, Denmark) ad libitum and had free access to tap water. The food supply was withdrawn 12 h before the animals were anaesthetized. Immediately before entering the experiment, the animals underwent a clinical examination. Following challenge/placebo inoculation, the animals were housed in individual pens.

**Bacterial inoculate.** *S. aureus* strain S54F9, originally isolated from a chronic embolic porcine lung abscess and previously used for experimental inoculation of pigs (18) was used. The strain was propagated for 18 h at 37°C in Luria-Bertani (LB) broth (19) with shaking, sedimented by centrifugation at 3000 xg for 30 min and resuspended in sterile isotonic saline. The viable count was determined by the plate count method (18) and the suspension was diluted with sterile isotonic saline to obtain a suspension containing 10⁸ colony-forming units (CFU)/ml.

**Experimental protocol.** The pigs, which remained clinically healthy during the acclimatization period, were randomly assigned into four groups. Each group contained three inoculated and one control animal (Table I). Following sedation by a procedure described recently (18), a catheter (22G) was inserted in the left ear vein for inoculation of 1x10⁸ bacteria/kg BW once (groups 1 and 2) at the beginning of the experiment (0 h) or twice (groups 3 and 4) at 0 h and 12 h (Table I). The pigs were euthanased at 6 h (group 1), 12 h (group 2), 24 h (group 3), or 48 h (group 4). Placebo-inoculated animals were given sterile saline. At termination of the experiment, the animals were euthanased by an overdose of 20% pentobarbital intravenously.

The protocol was approved under the Danish Animal Experimental Act (licence No. 2008/561-37).

**Postmortem assessment.** Following euthanasia, the animals were necropsied and tissues were sampled from gross lesions. Systematic sampling of tissues included specimens of the lung taken from margo dorsalis of the left diaphragmatic lobe together with the costo-chondral area of two ribs (nos. 8 and 9 on the right side), the cranial growth plate area of two thoracic vertebrae (nos. 8 and 9) and the sacral bone. The distal growth plate area of the right femoral, tibial, humeral, ulnar, and radial bones were also collected (Table I). All bones were trans-sectioned aseptically by a sagittal cut using an oscillating saw (IM-MAX MEDICAL, Frederiksborg, Denmark) before tissues were sampled. Tissues were fixed in 10% neutral buffered formalin for three days, processed through graded alcohols and xylene, and embedded in paraffin wax. Following fixation, the osseous tissues were decalcified in a solution containing 3.3% formaldehyde and 17% formic acid for 2 weeks. Sections of 4-5 μm were cut and stained with haematoxylin and eosin (H&E), and in selected cases by phosphotungstic acid haematoxylin (PTAH) and van Giesson for the demonstration of fibrin and collagen, respectively (20).

**Immunohistochemistry.** Immunostaining of different antigens was carried out on 4-5 μm tissue sections mounted on adhesive glass (Thermo Scientific, Menzel GmbH & CoKG, Baunschweig, Germany). An indirect immunostaining technique based on a specific *S. aureus* murine monoclonal antibody (ab37644; Abcam plc, Cambridge, UK) was used for the in situ identification of *S. aureus* bacteria. Additional indirect immunostains were applied for the demonstration of endothelial, mononuclear, polymorph nuclear, and epithelial cells (only on lung tissue) by the use of antibodies towards von Willebrand factor (A0082; Dako, Glostrup, Denmark), lysozyme (A0099; Dako), macrophages (MAC874G; AbD Serotec; MorphoSys UK Ltd, Oxford, UK), and cytokeratin (M 3515; Dako), respectively as the primary reagents. The immunostainings were performed using various antigen retrieval procedures and the application of the UltraVision LP Detection System HRP or for lysozyme Ultra Vision One Detection System (HRP) (Lab Vision Corporation, Fremont, CA, USA). Briefly, following deparaffinization, antigen retrieval procedures were carried out by treatment with 0.1% trypsin (Sigma-Aldrich Denmark A/S, Vallensbæk Strand, Denmark) solution for 15 to 20 min at 37°C (ab37644 and A0099), 0.018% or 0.072% protease (Sigma-Aldrich Denmark A/S) solution for 5 to 60 min (A0082 and M3515); Tris-EDTA boiling (Sigma-Aldrich Denmark A/S), pH 9.0 for 10 min (MAC874G) in a microwave oven (700 W). This was followed by blocking of endogenous peroxidase by 0.6% or 3.0% H₂O₂ for 15-
20 min, and blocking of nonspecific binding sites by Ultra V Block (Lab Vision Corporation). Following incubation with the primary reagents (except for lysozyme), a primary antibody enhancer was applied. Horseradish peroxidase (HRP) polymer was applied and the reaction was developed with aminoethylcarbazol (AEC) Single Solution as described by the manufacturer (Lab Vision Corporation). For lysozyme, diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. Throughout the immunostaining protocol, with the exception of the step between blocking of nonspecific binding and the application of the primary reagent, the slides were washed in Tris-buffered saline. Following immunostaining, the sections were counterstained with Mayer’s haematoxylin. Negative controls for the immunostainings were run on parallel sections without the primary reagent and with a nonsense polyclonal or monoclonal (matching isotype) antibody of the same concentration as the primary reagent.

Apoptosis. The presence of cell death due to apoptosis in osteomyelitic lesions was examined by use of the TdT-FragEL™ DNA Fragmentation Kit (Calbiochem, Gibbstown, NJ, USA). The assay was applied according to the instructions of the manufacturer (Lab Vision Corporation). For lysozyme, diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. Throughout the immunostaining protocol, with the exception of the step between blocking of nonspecific binding and the application of the primary reagent, the slides were washed in Tris-buffered saline. Following immunostaining, the sections were counterstained with Mayer’s haematoxylin. Negative controls for the immunostainings were run on parallel sections without the primary reagent and with a nonsense polyclonal or monoclonal (matching isotype) antibody of the same concentration as the primary reagent.

Microbiology. Tissues from the lungs and the distal metaphyseal area of the left femur were sampled for quantitative bacteriology. The lung tissue was collected from the dorsal margin of the left cranial lung lobe, whereas bone marrow tissue was drilled out using a 13 mm drill sterilised in boiling water and mounted on an electrical drill. Approximately 1 g of lung tissue and bone marrow was weighed and homogenized in 9 ml sterile isotonic saline using a stomacher (Stomacher lab-blender; Seward Medical, London, UK). Ten-fold dilutions in sterile isotonic saline of the homogenized tissues were prepared. From each of these preparations, 10 μl were inoculated on LB agar medium and incubated for 24 h at 37°C before counting the colonies. Counts per g were calculated. Colony morphology was evaluated and representative colonies were subcultured on blood agar (Blood agar base, CM55; OXoid, Basingstoke, Hampshire, UK) containing 5% sterile blood and phenotypically characterized using API ID 32 Staph (Biomerieux, Inc., Marcy-l’Etoile, France).

Statistics. The correlation over time between CFU/g of lung and bone tissue was tested by a linear regression analysis using logarithmic (base 10) transformed bacterial counts and using the computer program SAS/STAT™ (SAS Institute Inc., Cary, NC, USA). The significance level was set at p<0.05.

Results

The lungs of animals in groups 1 and 2 were free of lesions with the exception of a 2 mm in diameter haemorrhagic process localized in the left cranial lung lobe of one of the group 2 animals. In groups 3 and 4, two animals in each group had multiple, disseminated pulmonary petechia. A single process 2-3 mm in diameter was found in the right diaphragmatic lobe of two pigs. At gross inspection, lesions were not observed on the cut surfaces of any of the bones.

For all the immunohistochemical protocols, the reactivity was in agreement with the structures towards which the primary reagents were raised (S. aureus, endothelial, epithelial, mononuclear and polymorph nuclear cells), and all controls were negative.

Histologically, lesions in the form of microabscesses developed progressively in both the lung and metaphyseal...
tissues (Table I). In the systematic sampled lung tissues from margo dorsalis, lesions initiated as a few diminutive accumulations of neutrophils, sometimes co-localized with thrombosed capillaries within the alveolar septa, in animals inoculated for 6 h (group 1). After 12 h (group 2), multiple microabscesses had formed, and often they erupted into the alveolar lumen. In group 3 animals, given a second inoculation at 12 h and euthanased after 24 h, multiple microabscesses were also present (Figure 1). The centre of many microabscesses contained necrotic neutrophils and was
surrounded by a few macrophages and fibrin filaments. In group 4 animals, which also received two inoculations, pulmonary microabscesses were not found; however, within some bronchioles, neutrophils and mononuclear cells were present, along with an accumulation of mucus. The lung lesions of animals in groups 1 to 3 intensified with respect to hyperaemia, enhanced lymph flow seen as distended interstitial lymph vessels, and interstitial oedema. In some of the lung specimens, small thrombi without infiltration of inflammatory cells were also present within the walls of alveoli. Characteristically, within the lung lesions of group 1 to 3 animals, only a few bacteria were identified immunohistochemically. In contrast, immunopositive bacteria were not disclosed within the lung tissue of group 4 animals. The few processes observed macroscopically within the lungs of single animals in groups 2, 3, and 4 all were confirmed histologically to be non-encapsulated abscesses.

Bone lesions were absent during the first 6 h of infection, whereas they were observed as slight suppuration and/or microabscesses in bones of all animals infected for 12 h and

Figure 5. Tibia from a pig infected for 48 h with S. aureus shows the junction zone between the growth plate (GP) and the metaphysis (MP). Until the infiltration of neutrophils, the capillary loops were laid with normal endothelial cells (→), whereas they were destroyed when an influx of neutrophils had occurred (➢). Immunostained for von Willebrand factor. Bar=40 μm.

Figure 6. Tibia from a pig infected for 48 h with S. aureus. Simultaneously with the initiation of infection, fibrin was deposited (arrows) at the area of the capillary loops between the growth plate (GP) and the metaphysis (MP). PTAH. Bar=80 μm.

Figure 7. Femur from a pig infected for 48 h with S. aureus. Bacteria and inflammatory cells extend from the inflamed metaphyses (MP) into an intraphysal channel (arrow) of the growth plate (GP) next to an area of dead cartilage. Immunostained for S. aureus. Bar=160 μm.

Figure 8. Mean counts and standard deviation (SD) of S. aureus (CFU/g) in lung and femoral metaphysal tissues at 6 h, 12 h, 24 h, and 48 h after intravenous inoculation.
more (Table I). Although inflammatory lesions were not established at 6 h after challenge, single bacteria were regularly observed within the deep part of the metaphyses of long bones in group 1 animals. Along with the presence of suppuration and microabscess formation, thrombosis, oedema, and apoptotic osteosis were also regularly observed (Figure 2). In the group 2 animals (euthanased 12 h following a single injection of \textit{S. aureus}) the foci of suppuration had the shape of microabscesses and in several of these lesions, bacteria, both as single organisms and as colonies, were found within and around the capillary loops at the point of endochondral ossification, i.e. at the zone of chondral calcification beneath the growth plate (Figure 3). This pattern of suppuration and microabscess formation intensified in group 3 and 4 animals (inoculated twice (0 h and 12 h) and euthanased after 24 h and 48 h respectively). In these animals, it was also apparent that the infection and accompanying inflammatory reaction spread from its initiation deep in the metaphysis towards the capillaries next to the cartilage of the growth plate (Figures 3 and 4). Along with the influx of neutrophils in the area of capillary loops, destruction of the endothelial cells and deposition of fibrin were seen (Figures 5 and 6). In some of the long bones, but especially in femur and radius, areas of suppuration and microabscess formation were also present next to the resting zone of the growth plate, i.e. in the epiphysis. In accordance with these observations, bacteria (single or colonies) accompanied by suppuration were sometimes observed within the transphyseal vascular channels (Figure 7). Although osteomyelitis lesions were predominantly present in the long bones of the limbs (especially of the hind legs) some animals challenged for 12 h or more also revealed microabscesses in the costo-chondral junction of the ribs sampled. By contrast, lesions in the shape of microabscesses were not present in the vertebral column. However, areas of more diffuse infiltration with neutrophils were observed within the medulla cavity of vertebral bones and os sacrum in groups 3 and 4. Along with such infiltrations, only few bacteria were disclosed.

The bacterial counts within the lung and femur of animals from the four groups are shown in Figure 8. The linear correlation coefficient for the decline (from $1.5 \times 10^4$ to $1.8 \times 10^2$ CFU/g) of lung counts of \textit{S. aureus} was 0.748933 ($p=0.0051$), and for the increase of bacterial counts within the femoral metaphysis (from $1 \times 10^3$ to $1.5 \times 10^6$ CFU/g) the correlation coefficient was 0.611 ($p=0.0348$). Lung and bone tissues of the placebo-inoculated animals were sterile and free of lesions.

**Discussion**

The present study demonstrates that intravenous inoculation of pigs with \textit{S. aureus} results in the development of acute, suppurative pneumonia and osteomyelitic lesions (micro-abscesses). Microabscesses were present as early as 6 h after inoculation in the lungs, whereas they first became apparent after 12 h in the metaphysis of especially long bones (Table I). This propensity for an early development of lung lesions following intravenous inoculation of \textit{S. aureus} is in accordance with observations in a recently published experimental, systemic short-term study (2 h to 6 h) in pigs (18). In both the lungs and bones, lesions increased in numbers and size over time, however, the pulmonary microabscesses had disappeared after 48 h. On the contrary, the infection and accompanying inflammatory reaction progressed until the end of the experiment (48 h) in several bones.

In pre-pubertal children, haematogenous osteomyelitis involves mostly the metaphysis of long bones, especially of the femur and tibia, and in most cases, lesions are present as a single focus (3, 21). By contrast, osteomyelitis in adults most frequently involves the vertebral bodies (3). This age-related pattern of human osteomyelitis is similar to the observations in the present model in juvenile pigs, in which the long bones were most frequently affected, whereas microabscesses were not present in the bones of the spine (Table I). Moreover, in the present model the infection initiated deep in the metaphyses, from which it subsequently spread to the area underlying the growth plate, i.e. to the capillary loops. These loops are sites predisposed to bacterial embolization in neonatal sepsis of animals (22). This also parallels the situation in children, where osteomyelitic lesions typically are focal and localized adjacent to the growth plate (21). Apart from the unique anatomical structures at the point of endochondral ossification, specific binding of \textit{S. aureus} to the cartilage surface is also thought to promote initiation of osteomyelitis in this area (9). From the present observations, it is therefore also likely that the destruction of capillary loops will accentuate this localization of \textit{S. aureus}. Finally, the observation of transphyseal spread of the infection through the vascular channels of the growth plate is an important complication in human osteomyelitis (23), as well as in spontaneous osteomyelitic lesions of pigs (6).

Devitalization of bone tissue is an important feature of osteomyelitis in both humans (3) and pigs (6). The pathogenesis of osteitis in osteomyelitic lesions is complex, and it may be established due to a number of causes such as compression and obliteration of vascular channels, decline in oxygen tension, and release of different mediators active during acute inflammation (e.g. prostaglandins, tumour necrosis factor, and transforming growth factor) (21, 24). In accordance with the observation of apoptotic osteogenic cells within dead bone tissue, it was recently found that murine osteoblasts containing \textit{S. aureus} express tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which potentially will cause bone destruction (25).

In the present model, the pulmonary inflammatory response and the bacterial load of the lungs declined during
the infection period of 48 h. This is in contrast to the lesions and bacterial burden of bones, which increased significantly. An explanation for this might be the presence of pulmonary intravascular macrophages (PIMs), which are known to promptly phagocyte *S. aureus* following haematogenous spread to the lungs (26). Activation of these cells by the first inoculation could also explain the efficient pulmonary elimination of the bacteria from the second inoculation, and thereby the lack of lung lesions 48 h after challenge.

The incidence of human osteomyelitis due to *S. aureus* has increased dramatically in recent years (1) and in all types of osteomyelitis in humans, *S. aureus* remains the major pathogen (27). In the late stage of septicemia of humans, which is most frequently caused by *S. aureus* strains in nosocomial infections (28), bacterial seeding of different organs, including bones resulting in the development of osteomyelitis (29) is common. Only poor results are achieved with antimicrobial therapy of bone infections (30-32). Infections with antibiotic-resistant *S. aureus* have put further focus on the risk of a negative outcome of therapy leading to pathological fractures and other disabilities, which have also increased in recent years (2).

Apart from the similarities in pathogenesis and pathology of the present model to human osteomyelitis, the development of porcine models of bacteraemia, septicemia, and pyaemia possesses a number of advantages when comparing the disease entity with the situation in humans. In particular, the size of the pig and its anatomy, nutrient requirements, metabolic rate, general physiological behaviour, pattern of organ development, and capillary density makes it the animal species of choice (15, 16, 33). The size is important because this will allow for improved monitoring of the models through i) repeated sampling of e.g. blood, body-fluids and tissue specimens, ii) application of non-invasive diagnostic techniques (e.g. PET- and CT-scanning, and echocardiography), and iii) assessment of e.g. neurological deficiencies as used for human patients. Refinement of surgical procedures (e.g. excision of abscesses, transplantation, and application of prostheses/medical devices) will also be feasible in a porcine model.

In conclusion, the present porcine model seems useful as a model of the frequent osteomyelitic lesions which occur especially in pre-pubertal children following haematogenous spread of *S. aureus*. Therefore, this model should be reliable in studies of this disease with respect to pathophysiology, pathomorphology, strain virulence, and therapy.

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**References**


