EXPERIMENTALLY INDUCED DISEASE

Pathology and Biofilm Formation in a Porcine Model of Staphylococcal Osteomyelitis


*Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen,
†Department of Pathology and Wildlife Diseases, Veterinary Institute, Uppsala, Sweden,
‡Department of Small Animal Clinical Sciences, Faculty of Life Sciences, University of Copenhagen,
§Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen and
||Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark

Summary

A porcine model was used to examine the potential of human and porcine Staphylococcus aureus isolates to induce haematogenously spread osteomyelitis. Pigs were inoculated in the right femoral artery with one of the following S. aureus strains: S54F9 (from a porcine lung abscess; n = 3 animals), NCTC-8325-4 (a laboratory strain of human origin; n = 3 animals) and UAMS-1 (a human osteomyelitis isolate; n = 3 animals). Two pigs were sham inoculated with saline. At 11 or 15 days post infection the animals were scanned by computed tomography before being killed and subjected to necropsy examination. Osteomyelitis lesions were present in the right hind limb of all pigs inoculated with strain S54F9 and in one pig inoculated with strain NCTC-8325-4. Microscopically, there was extensive loss of bone tissue with surrounding granulation tissue. Sequestrated bone trabeculae were intermingled with colonies of S. aureus as demonstrated immunohistochemically. By peptide nucleic acid fluorescence in situ hybridization bacterial aggregates were demonstrated to be embedded in an opaque matrix, indicating that the bacteria had formed a biofilm. Development of experimental osteomyelitis was therefore dependent on the strain of bacteria inoculated and on the formation of a biofilm.

© 2012 Elsevier Ltd. All rights reserved.

Keywords: biofilm; osteomyelitis; pathology; pig model; Staphylococcus aureus

Introduction

Staphylococcus aureus is the most common cause of haematogenously spread osteomyelitis (HO), a disease which occurs primarily in children (Lew and Waldvogel, 1997). An episode of bacteraemia leads to settling of bacteria in bones and initiation of a focus of osteomyelitis (Lew and Waldvogel, 1997). A reliable animal model of HO should therefore be based on inoculation of bacteria directly into the bloodstream. A variety of such models has been described (Table 1). Some models use bacterial strains isolated from the same animal species, while others use human strains sometimes isolated from patients with osteomyelitis. The origin of the bacterial strain is likely to influence the outcome of the lesions induced in a model, due to potential host specificity and differential expression of virulence factors (Sung et al., 2008; Wright and Nair, 2010). One of the main virulence factors associated with the development of staphylococcal osteomyelitis, and in particular the development of chronic disease, is the formation of a bacterial biofilm (Gristina et al., 1985; Brady et al., 2008). Biofilms develop on inert surfaces or dead tissue and constitute a protected environment for the bacteria in which they may avoid the effects of antibiotics and host immune defence (Hoiby et al., 2010). The biofilm itself therefore induces an ongoing inflammatory reaction (Costerton et al., 1999).

Intra-arterial inoculation of pigs with a lower number of a porcine S. aureus strain (S54F9) than used in other animal models of HO results in the development
Table 1
Animal models of osteomyelitis based on haematogenous injection of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Animal species</th>
<th>Bone trauma or artificial necrosis</th>
<th>Strain of <em>S. aureus</em></th>
<th>Origin</th>
<th>CFU injected</th>
<th>Osteomyelitis frequency (%)</th>
<th>Primary systemic effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>Rabbit</td>
<td>No</td>
<td>Micrococcus</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Sepsis and non-osseous abscesses</td>
<td>Rodet (1884), 1973</td>
</tr>
<tr>
<td>Rabbit, dog</td>
<td>No</td>
<td>Attenuated <em>S. aureus</em></td>
<td>Human osteomyelitis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>None</td>
<td>Lexer, 1894</td>
</tr>
<tr>
<td>Dog</td>
<td>No</td>
<td>NR</td>
<td>Human and canine</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Sepsis</td>
<td>Starr, 2002</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Yes</td>
<td>NR</td>
<td>Human osteomyelitis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Sepsis and non-osseous abscesses</td>
<td>Thompson and Dubos, 1938</td>
</tr>
<tr>
<td>Rabbit (immunized)</td>
<td>No</td>
<td><em>S. aureus</em></td>
<td>Human osteomyelitis</td>
<td>NR</td>
<td>NR</td>
<td>38</td>
<td>Non-osseous abscesses</td>
<td>Weaver and Tayler, 1943</td>
</tr>
<tr>
<td>Rabbit†</td>
<td>No</td>
<td><em>S. aureus</em></td>
<td>Human osteomyelitis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>None</td>
<td>Kadyrov et al., 1966</td>
</tr>
<tr>
<td>Rabbit†</td>
<td>No</td>
<td><em>S. aureus</em></td>
<td>Avian infection</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>None</td>
<td>Koulunderliev, 1971</td>
</tr>
<tr>
<td>Rabbit†</td>
<td>No</td>
<td><em>S. aureus</em></td>
<td>Avian infection</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>None</td>
<td>Holland, 1972</td>
</tr>
<tr>
<td>Chicken</td>
<td>No</td>
<td>6/42E/53/77/83A/84†</td>
<td>Avian infection</td>
<td>10^7</td>
<td>100</td>
<td>None</td>
<td>Emслиe and Nade, 1983</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>No</td>
<td>6/42E/53/77/83A/84†</td>
<td>Avian infection</td>
<td>10^5−10^6</td>
<td>100</td>
<td>None</td>
<td>Speers and Nade, 1985</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>No</td>
<td>6/42E/53/77/83A/84†</td>
<td>Avian infection</td>
<td>10^5</td>
<td>NR</td>
<td>NR</td>
<td>Alderson et al., 1986</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Yes</td>
<td>ATCC-25932</td>
<td>Laboratory strain</td>
<td>10^7</td>
<td>100</td>
<td>Not examined</td>
<td>Whalen et al., 1988</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Yes</td>
<td>ATCC-25932</td>
<td>Laboratory strain</td>
<td>10^7</td>
<td>100</td>
<td>Not examined</td>
<td>Morrissey and Haynes, 1989</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>No</td>
<td>Type 8 capsular isolate</td>
<td>Human arthritis</td>
<td>10^7</td>
<td>100</td>
<td>None</td>
<td>Daum et al., 1990</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Yes</td>
<td><em>S. aureus</em> Phillips</td>
<td>Human osteomyelitis</td>
<td>10^6</td>
<td>100</td>
<td>None</td>
<td>Hien et al., 1995</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>M130</td>
<td>Human infection</td>
<td>10^6</td>
<td>100</td>
<td>None</td>
<td>Hien et al., 1995</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Yes</td>
<td>LS-1</td>
<td>Mouse pathogen</td>
<td>10^7</td>
<td>100</td>
<td>Not examined</td>
<td>Chada et al., 1999</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Yes</td>
<td>LS-1</td>
<td>Mouse pathogen</td>
<td>10^7</td>
<td>100</td>
<td>Not examined</td>
<td>Yoon et al., 1999</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-1</td>
<td>Human osteomyelitis</td>
<td>10^7</td>
<td>70</td>
<td>Not examined</td>
<td>Elasri et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-237</td>
<td>(Mutation of UAMS-1)</td>
<td>10^7</td>
<td>5</td>
<td>Not examined</td>
<td>Elasri et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>RN6390</td>
<td>NCTC-8325-4, human infection</td>
<td>10^8</td>
<td>100</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-957</td>
<td>Human osteomyelitis</td>
<td>10^8</td>
<td>44.4</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-969</td>
<td>(Mutation of UAMS-1)</td>
<td>10^8</td>
<td>96.3</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>RN6290</td>
<td>(Mutation of UAMS-1)</td>
<td>10^8</td>
<td>61.8</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>RN6290</td>
<td>(Mutation of UAMS-1)</td>
<td>10^8</td>
<td>37</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-930</td>
<td>(Mutation of UAMS-1)</td>
<td>10^8</td>
<td>18.7</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>No</td>
<td>UAMS-969</td>
<td>(Mutation of UAMS-1)</td>
<td>10^8</td>
<td>90</td>
<td>Not examined</td>
<td>Blevins et al., 2003</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>No</td>
<td>S54F9</td>
<td>Porcine lung abscess</td>
<td>10^8</td>
<td>100</td>
<td>Non-osseous microabscesses</td>
<td>Jensen et al., 2010</td>
<td></td>
</tr>
</tbody>
</table>
of osteomyelitis only in bones supplied by the artery into which the injection is made (Johansen et al., 2011). The aims of the present study were (1) to compare the infection potential of the porcine strain (S54F9) with two S. aureus strains of human origin in this model and (2) to examine the development of HO with a focus on pathology and the localization and microenvironment of S. aureus.

Materials and Methods

Animals

Eleven healthy 12-week-old female Yorkshire-Landrace-cross pigs, with a body weight (BW) of approximately 30 kg were obtained from a specific pathogen free (SPF) herd (Harris and Alexandra, 1999). On arrival, the animals were allowed to acclimatize for 14 days. The animals were fed a commercial pig diet (Svine Erantis Brogaarden ApS, Lyng, Denmark) ad libitum and had free access to tap water. The Danish Animal Experimental Act approved the protocol (licence number 2008/561-37).

Experimental Design

The animals were randomly assigned into four groups (A, B, C and D) housed in separate pens (Table 1). The control group of two animals (group A) was inoculated with saline while the three infected groups of three animals each were inoculated with S. aureus strains S54F9 (group B), NCTC-8325-4 (group C) or UAMS-1 (group D). Premedication was performed with an intramuscular injection (1 ml/10 kg BW) of a solution containing a mixture of 125 mg zolezepam, 125 mg tiletamine, 6.25 ml xylazine (20 mg/ml), 1.25 ml ketamine (100 mg/ml), 2 ml butorphanol (10 mg/ml) and 2 ml metadon (10 mg/ml). A catheter was inserted into a lateral ear vein and anaesthesia was maintained with propofol (10 mg/ml) 1 ml/kg BW/h. While anaesthetized, the pigs were inoculated in the direction of the blood flow into the right femoral artery. The animals were killed at 11 or 15 days post inoculation (dpi) by 20% pentobarbital given intravenously. Blood samples for screening of bacteremia were taken from the jugular vein prior to inoculation, 20 min after inoculation and again at 1, 7, 10 and 15 dpi.

Bacterial Isolates

The S. aureus strains of human origin were UAMS-1 and NCTC-8325-4, which have been used previously for induction of osteomyelitis in mice (Blevins et al., 2003). NCTC-8325-4 is derived from a case of human sepsis (Cassat et al., 2005). UAMS-1 is a human
osteomyelitis isolate that has also become a preferred strain for studying the virulence of *S. aureus* (Cassat et al., 2005). The *S. aureus* isolate S54F9 was obtained from a chronic embolic pulmonary abscess in a Danish slaughter pig (Leifsson et al., 2010). The strains of *S. aureus* were prepared as described by Johansen et al. (2011) and diluted with sterile isotonic saline 0.9% to obtain an inoculation dose of 10,000 colony forming units (CFU)/kg BW in a volume of 1 ml.

**Clinical Examination**

All animals underwent clinical examination during the acclimatization period and subsequently twice daily after inoculation. In cases of clinical signs of disease, the frequency of clinical examinations was increased and lame animals were treated with intramuscular injections (0.1 mg/kg BW) of buprenorphin (Temgesic 0.3 mg/ml; Schering-Plough, Heist-op-den-Berg, Belgium) every 6 h. The BW of the pigs was measured before inoculation and at 8 dpi.

**Blood Samples**

For screening of bacteraemia, 4 ml blood samples were collected and analysed as previously described (Johansen et al., 2011).

**Computed Tomography**

The location of osteomyelitic lesions was evaluated by computed tomography (CT). All pigs were scanned under anaesthesia (see Experimental Design) to prevent movement, before inoculation and again immediately after death. The scanning was performed with a single slide CT scanner (Siemens Somatom Emotion, Erlangen, Germany). The pigs were positioned in ventral recumbency with the hind limbs pulled caudally and scanned in the craniocaudal direction from the hip to the digits with a slide thickness of 5 mm (kV = 130 and mAs = 55). The scans were reconstructed using a standard soft tissue algorithm (B40s) and evaluated on a dedicated workstation (Siemens Leonardo, Erlangen, Germany).

**Pathology**

The femoral and tibial bones were sectioned sagittally and scored using a classification system modified from that described by Rissing et al. (1985); 0, no lesion; 1, abscessation; 2, abscessation with penetration through the cortical bone. Samples of the dorsal border of the left diaphragmatic lung lobe, right kidney, spleen and liver were taken for microscopical examination. Samples from the distal growth plate of the right and left femur and the proximal growth plate of the right and left tibia were collected using an oscillating saw (IM-MAX MEDICAL, Frederiksberg, Denmark). Due to the fragility and small size of the bones in the distal hindlimbs (distal to the tibial diaphysis), these were fixed in toto. All tissues were fixed in 10% neutral buffered formalin for 3 days, after which the osseous tissues were decalcified (Johansen et al., 2011). After decalcification, bones of the distal hindlimbs were sectioned sagittally and scored as described above.

After fixation and/or decalcification, tissues were processed routinely and embedded in paraffin wax. Sections (4–5 μm) were stained with haematoxylin and eosin (HE). Immunohistochemistry (IHC) was used for in situ identification of *S. aureus*. The primary antibody was a murine monoclonal antibody specific for *S. aureus* (ab37644; Abcam, Cambridge, UK) (Johansen et al., 2011).

**Microbiology**

Tissue samples from the lungs and the distal metaphyseal area of the right and left femur and the proximal metaphyseal area of the right and left tibia were also collected for quantitative bacteriology. Metaphyseal bone marrow tissue without gross lesions was drilled out with a 13 mm drill bit sterilized in boiling water and mounted on an electrical drill. The samples were analysed as described by Jensen et al. (2010). Swabs were taken from the bone abscesses and streaked on Luria-Bertani (LB) agar and incubated for 24 h at 37°C. All isolates were characterized using API ID 32 Staph (Biomerieux Inc., Marcy-l’Etoile, France).

**Peptide Nucleic Acid Fluorescence In situ Hybridization**

Paraffin wax-embedded femoral bone lesions from all pigs of group B and pig C2 were selected for peptide nucleic acid fluorescence in situ hybridization (PNA FISH). Sections (4 μm) were mounted on adhesive slides (Superfrost R plus, Menzel-Glaser, Germany) and dewaxed (Bjarnsholt et al., 2009a). A Texas red-labelled, *S. aureus*-specific PNA probe in hybridization solution (AdvanDx Inc., Woburn, Massachusetts, USA) was added to each section and hybridized on a PNA FISH workstation at 55°C for 90 min. The slides were washed for 30 min at 55°C in wash solution (AdvanDx Inc.). VECTAShIELD mounting media with 4’,6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, California, USA) was applied and a cover slip was fixed to each slide. Slides were examined using a fluorescence microscope equipped with a Texas red and DAPI filter (Bjarnsholt et al., 2009a).
Results

Clinical Observations
All animals were healthy prior to inoculation. At 5 dpi all pigs in group B showed lameness of the inoculated limb. These animals displayed pain on palpation of the affected bones and had intermittent mildly elevated body temperature. Clinical signs were not observed in pigs of groups A, C and D. Although pigs from group B had a lower weight gain compared with the other groups, all animals gained weight during the experiment.

Blood Samples
No bacteria were isolated from blood at any time point of the experiment.

Computed Tomography
Bone changes were not observed in the hindlimbs of any animal prior to inoculation. At the end of the experiment, no changes were seen in the non-inoculated (left) hindlimb, nor in pigs of group A (controls). In the inoculated (right) hindlimb, changes were only present in animals of group B and in pig 2 from group C (Fig. 1). These changes consisted of osteolytic areas in which one or more central foci of increased opacity were identified. Observations on individual animals are described below.

Pig B1: Distally in the femur, a lesion extended from the metaphysis and growth plate centrally in the bone to the medial aspect of the distal epiphysis (i.e. the medial femoral condyle) (Fig. 2). In the proximal tibia from the metaphysis to the epiphysis, including the growth plate, a lesion was present only in the trabecular bone. In the distal part of the third metatarsal bone a small osteolytic lesion was noted within the trabecular and cortical bone and an associated soft tissue swelling was present.

Pig B2: Two lucent areas were seen in the femur. The lesions were situated medially in the trabecular bone of the metaphysis with extension through the growth plate and just into the epiphysis. In one of the lesions further medial extension into the cortical bone of the metaphysis was seen.

Pig B3: Distally in the femur, a lesion was observed at the medial aspect of the metaphysis in close approximation to the growth plate. A second lesion was present at the level of the lateral femoral condyle in the trabecular bone and the dorsal aspect of the cortical bone. Two tibial lesions were observed in the medial and lateral aspect of the proximal metaphysis and the growth plate and epiphysis, respectively. One of these lesions showed further medial extension into and through the cortical bone of both the metaphysis and epiphysis. In the distal part of calcaneus and in the fourth and fifth metatarsal bones, small osteolytic lesions with associated soft tissue swellings were observed.

Pig C2: In the distal femur a trabecular osteolytic area was observed in the plantar aspect of the metaphysis close to the growth plate. A comparable lesion was recognized proximally in the tibia.

Gross Pathology
Apart from bone changes observed by CT, other lesions were not detected grossly. The scoring of lesions in the right femoral, tibial, tarsal and metatarsal bones is presented in Fig. 3. All bones of the left hindlimbs (non-inoculated leg) were scored 0. In group B the inflammation sometimes penetrated into and through the cortical bone, leading to the formation of soft tissue abscesses (Fig. 4A), Femoral abscesses of animals B1 and B3 extended through the articular cartilage, leading to secondary fibrinous and purulent arthritis in the stifle joints. Soft pink
Fig. 2. CT scanning of the femoral lesion (right inoculated limb) in animal B1. (A) The different planes in which the lesions were visualized. Red, sagittal plane; green, transverse plane. (B) Sagittal plane. An osteolytic area extends from the metaphysis and growth plate centrally in the bone to the medial aspect of the distal epiphysis (i.e. the medial femoral condyle). In the metaphysis and growth plate the lesion was irregular and the normal contour of the medial cortex cannot be seen. In the medial femoral condyle, both trabecular and cortical bone are involved. (C) Transverse plan, 10 mm sections. Multiple central foci of increased opacity are seen throughout the lesion. No lesions are present in the non-inoculated left limb.

Score 2

<table>
<thead>
<tr>
<th>B1</th>
<th>B1</th>
<th>B1</th>
</tr>
</thead>
</table>

Score 1

<table>
<thead>
<tr>
<th>B2</th>
<th>B2</th>
</tr>
</thead>
</table>

Score 0

| A1 | A1 | A1 | A1 | B1 | B2 | C1 | C1 | C1 | C1 | D1 | D1 | D1 | D1 |

| A2 | A2 | A2 | A2 | B2 | C2 | C3 | C3 | C2 | C2 | D2 | D2 | D2 | D2 |

| F | Ti | T | M | F | Ti | T | M | F | Ti | T | M | F | Ti | T | M |

Group A

Control (saline)

n = 2

Group B

S54F9 (porcine)

n = 3

Group C

NCTC8325-4 (human)

n = 3

Group D

UAMS-1 (human)

n = 3

Fig. 3. Gross bone lesion score in the right hindlimb of pigs inoculated with saline (group A) or three different strains of S. aureus (groups B, C and D) into the right femoral artery. Bone lesions of: F, femur; Ti, tibia; T, tarsus; M, metatarsus. Scores: 0, no lesion; 1, abscessation; 2, abscessation with penetration through cortical bone. In cases where two lesions were seen in the same bone, only the highest score is included in the figure.
granulation tissue surrounded the purulent material of all lesions; however, this was most pronounced in animals B2 and C2. In the femoral abscesses of animal B2, granulation tissue and surrounding dense fibrotic tissue was present. Gross lesions were not observed in the spleen, liver, lungs or kidneys.

**Histopathology**

One microabscess in the distal metaphyseal area of the fifth left metatarsal bone in animal B1 was detected microscopically in addition to the lesions identified grossly. All bone abscesses contained central neutrophils surrounded by granulation tissue. Viable neutrophils (Fig. 4B) were intermingled with necrotic inflammatory and tissue cells collectively forming amorphous granular debris. Multiple fragmented and necrotic bone trabeculae, with empty lacunae, were often seen in the centre of abscesses (Fig. 4C). Sometimes, the necrotic trabeculae were surrounded by fibrin and numerous activated osteoclasts (Fig. 4C), while bone trabeculae outside the
granulation tissue were bordered by a layer of osteoblasts (Fig. 4D). Proliferation of fibroblasts and dense fibrosis made up the surrounding granulation tissue (Fig. 4D). The layer of granulation tissue associated with the femoral and tibial lesions of animals B2 and C2 was more prominent and thicker when compared with all other abscesses in group B animals. Fragments of dead primary spongiosa were also present among the neutrophils due to penetration of the growth plate by the abscesses (Figs. 4A, E and F). At sites of inflammatory extension through cortical bone and articular cartilage, the normal histological architecture of bone and cartilage was completely destroyed and replaced by neutrophils and necrotic debris. All bacteria in HE-stained sections (Fig. 4F) were identified by IHC as *S. aureus* (Fig. 5A). In the bone abscesses, bacteria were observed both as single organisms and in colonies, within and around the capillary loops at the point of endochondral ossification (Figs. 4F, 5A). Colonies of *S. aureus* were also detected centrally in bone and soft tissue abscesses (Fig. 4C). The spleen, liver and kidneys of all animals were free of microscopical lesions, with the exception of pig B1 in which a secondary, acute microabscess (a small focus of neutrophils) was seen in the lung.

**Microbiology**

*S. aureus* was re-isolated from all bone abscesses. Bacteriological examination of lung tissue revealed that only pig B1 had secondary embolic seeding of *S. aureus* (200 CFU/g lung tissue). *S. aureus* bacteria were detected only in bone tissue drilled out from animal B1 (900 CFU/g bone tissue).

**Peptide Nucleic Acid Fluorescence In situ Hybridization**

PNA FISH-positive *S. aureus* was identified in the femoral abscesses of all group B animals and pig C2. The bacteria were present as aggregates of small loosely-packed cocci. The aggregates were embedded in an opaque matrix suggesting that the bacteria had formed a biofilm (Fig. 5B).

**Discussion**

The virulence of three *S. aureus* strains of porcine and human origin was examined in a porcine model of HO. Bone lesions were present in three, one and none of the recipients of strains S54F9 (porcine), NCTC-8325-4 and UAMS-1 (human), respectively. An aggressive disease process with severe bone destruction and secondary soft tissue abscession is sometimes described in children with HO (Lew and Waldvogel, 1997). Comparable lesions were induced by the porcine strain (S54F9), but milder lesions mimicking subacute ‘Brodie abscesses’ (Steer and Carapetis, 2004) developed in pigs B2 and C2. Therefore, all animals with clinical signs of lameness and/or bone lesions developed HO in a similar pattern as described for human patients. One of the pigs (B1) that received the porcine strain showed acute secondary embolic seeding of *S. aureus* to the lungs and in the fifth left metatarsal bone. Sepsis following HO occurs rarely in paediatric cases (Nade, 1983).

CT scanning provides a superior non-invasive tool for studying bone abscesses in three planes, giving information on their exact anatomical location and size. In this pig model, the majority of bone abscesses were in the metaphyseal area, which corresponds to the most common location in children with HO (Lew and Waldvogel, 1997; Steer and Carapetis, 2004). This characteristic metaphyseal localization is related to the anatomical arrangement of the metaphyseal vessels beneath the growth plate (Trueta, 1959). Inside the osteomyelitis lesions there were colonies of bacteria within the metaphyseal

![Fig. 5. Lesions caused by *S. aureus* strain S54F9. (A) Identification of *S. aureus* bacteria (arrow) in the abscess shown in Fig. 4F. IHC. Bar, 80 μm. (B) Within the femoral abscess of pig B2 an aggregate of *S. aureus* bacteria (arrow) is embedded in an opaque matrix. PNA FISH. Bar, 10 μm.](image)
Porcine Model of Osteomyelitis

Capillary loops, so the present porcine model supports the theory that the pathogenesis of abscess formation in bones involves haematogenous spread. CT scanning has been reported as optimal for detection of small sequestra of devitalized bone tissue (Hernandez, 1983), which are reported to be present in paediatric osteomyelitis lesions after 10 days of infection (Steer and Carapetis, 2004). In the present study, devitalized bone tissue was seen as foci of increased opacity on the CT scans. In children, damage to the growth plate due to ischaemia or direct chondrolysis is also seen and associated with growth disturbance related to the volume of tissue destroyed and the location of the destruction (Nade, 1983). Some of the lesions in group B animals had an even more aggressive profile and penetrated through the growth plate into the epiphysis and further through the articular cartilage into the joint. Similar secondary arthritis is also reported in children (Nade, 1983; Steer and Carapetis, 2004). The disease process of children with HO therefore shows similarities with the pathogenesis and pathology of the lesions in the pigs of the present study.

The human strains UAMS-1 and a derivative of strain NCTC-8325-4 have been used previously in murine models of HO (Table 1). In these studies, animals received injections of $10^8$ CFU into the tail vein, and while the mice showed no clinical signs of disease, microscopical signs of osteomyelitis were seen in all mice at 14 dpi regardless of the strain (Blevins et al., 2003). However, in that study animals were not examined for primary systemic side-effects. In the present model, pigs inoculated with UAMS-1 did not develop osteomyelitis and only one pig of three inoculated with NCTC-8325-4 had osteomyelitis. In comparison to the murine model we used a much lower inoculation dose ($10^5$ CFU/kg BW). This inoculation dose was based on a previous porcine dose-response study using the porcine strain (Johansen et al., 2011). Presumably, host specificity of the strains is important for the outcome of the infection. Therefore, the inoculation dose of the human strains may need to be increased in order to observe the same lesions as for the porcine strain. In all previous studies, the intravenously inoculated models received bacterial doses above $10^8$ CFU to obtain a high frequency of osteomyelitis when inoculated with human strains.

In addition to host specificity, the pathogenesis of S. aureus osteomyelitis may also be associated with a variety of bacterial virulence factors including both structural and secreted products (Wright and Nair, 2010). However, the diversity of pathogenicity between the different S. aureus strains may arise from a diverse array of these virulence factors, which also may contribute to the different outcomes of the present study. The nature of the inoculated S. aureus strain and the species and individual resistance of the host determine the occurrence and progression of experimentally induced osteomyelitis, and it should be considered that animal models may sometimes be unreliable for prediction of bacterial virulence in infections because of host specificity. The strain and host should therefore be considered when comparing the different animal models of HO and in future studies based on the porcine model.

One of the main virulence factors associated with development of staphylococcal osteomyelitis, and in particular the development of chronic disease, is formation of a bacterial biofilm (Gristina et al., 1985; Brady et al., 2008). In cases where the bacteria succeed in colonization followed by biofilm formation within the host, the infection often turns out to be untreatable and becomes chronic (Brady et al., 2008; Hoiby et al., 2010). Numerous in vitro studies of biofilms have characterized a series of events that lead to biofilm formation (Gristina et al., 1985; Otto, 2008). These events include growth and proliferation of bacterial aggregates (microcolonies) embedded in a self-made, protective matrix of exopolymers (Gristina et al., 1985; Otto, 2008).

In the present study, bacterial aggregates were seen in situ within the devitalized bone tissue by PNA FISH after infection with strains S54F9 and NCTC-8325-4. The aggregation of bacteria was similar to previous observations in chronic biofilm-based infections such as cystic fibrosis (Bjarnsholt et al., 2009a), chronic wounds (Bjarnsholt et al., 2008) and infections related to permanent tissue fillers (Bjarnsholt et al., 2009b). Therefore, and in accordance with the presence of granulation tissue, the osteomyelitis lesions of the pigs developed into a pathological and microbiological chronic stage shortly after infection. This observation may explain why antibiotic therapy sometimes fails in patients diagnosed at an early stage of disease (Nade, 1983; Lew and Waldvogel, 1997; Hoiby et al., 2010). The resistance to antibiotics induced by biofilm formation is mediated through a very low metabolism and down-regulation of cell division by the entrenched bacteria (Brady et al., 2008). Furthermore, the biofilm acts as a diffusion barrier to slow down the infiltration of some antimicrobial agents (Brady et al., 2008). Together with the resistance raised by the biofilm, other features of S. aureus are connected with failure of antibiotic treatment. These include the intracellular location and presence of mutant forms such as small colony variants, both representing a further complication in the management of staphylococcal osteomyelitis (Wright and Nair, 2010). S. aureus, which is the most common aetiologic agent of osteomyelitis, is a well known potent biofilm-forming bacterium, leading to the perception
that biofilm formation is a critical step in the pathogenesis of osteomyelitis (Brady et al., 2008). The results of the present study support this theory of co-occurrence between biofilm formation and chronic osteomyelitis.

**Acknowledgements**

We thank B. Andersen and L. Kioerboe for excellent laboratory assistance. This work was financed by grant number 271-07-0417 from the Danish Medical Research Council, the Orthopedic Surgery Foundation in Aarhus, the Fund of King Christian X and the Beckett Foundation. No conflicts of interest are declared by the authors.

**References**


Matsushita K, Hamabe M, Matsuoka M, Aoki H, Miyoshi K et al. (1997) Experimental hematogenous...


Received, October 25th, 2011
Accepted, January 21st, 2012