Refinement of a hematogenous localized osteomyelitis model in pigs

Aage Kristian Olsen Alstrup[1], Karin Michaelsen Nielsen[2,3], Henrik Carl Schønheyder[4,5], Svend Borup Jensen[3,6], Pia Afzelius[7], Páll S. Leifsson[2] and Ole Lerberg Nielsen[2]

[1]*Department of Nuclear Medicine & PET-Centre, Aarhus University Hospital, Aarhus*

[2]*Department of Veterinary Disease Biology, University of Copenhagen, Copenhagen;*

[3]*Department of Nuclear Medicine, Aalborg University Hospital, Aalborg*

[4]*Department of Clinical Microbiology, Aalborg University Hospital, Aalborg*

[5]*Department of Clinical Medicine, Aalborg University, Aalborg*

[6]*Department of Chemistry and Biochemistry, Aalborg University, Aalborg*

[7]*Department of Diagnostic Imaging, Copenhagen University Hospital, North Zealand, Hillerød, Denmark.*

Correspondence: Aage Kristian Olsen Alstrup, DVM, Ph.D. & Associate Professor
Department of Nuclear Medicine & PET-Centre, Aarhus University Hospital
Nørrebrogade 44, 10C, DK-8000 Aarhus C, Denmark
Email: aagols@rm.dk

Summary

We have previously developed a model of localized osteomyelitis by injecting *Staphylococcus aureus* (*S. aureus*) unilaterally into the femoral artery of juvenile domestic pigs (*Johansen et al., 2012*; *Nielsen et al., 2015*). We used this model for the evaluation of bone-infection tracers applicable for positron emission tomography (PET) and single-photon emission computed tomography (SPECT) (*Nielsen et al., 2015*). However, several of the 40 kg pigs were euthanized prior to PET and SPECT scanning due to lameness, shallow respiration, fever and anorexia; the last three clinical signs indicating dissemination of *S. aureus* to the lungs and other internal organs. We therefore decided to refine our model in order to improve the success rate. We speculated that younger pigs might respond differently to inoculations. A total of ten female domestic pigs were included in our study, three with a body weight of 40 kg (± 1 kg) and seven with a body weight of 20 kg (± 1 kg). The pigs were born in a SPF facility and were raised in the same herd. Pigs were scored daily for pain, including lameness. From the onset of the first clinical signs we treated seven of the pigs (one 40 kg and six 20 kg) with procaine benzylpenicillin to which the bacterial strain was susceptible. Further treatment (analgesia by buprenorphine) or euthanasia depended on disease severity and took place due to fever and shallow respiration in the 40 kg pig-group and due to lameness and pain in hind limbs in the 20 kg pig-group. After two weeks, the remaining pigs were euthanized; necropsy was performed on all ten pigs. One of the 40 kg and five of the 20 kg pigs were euthanized before day 14. The necropsy results showed osteomyelitis caused by *S. aureus* in six of the seven 20 kg pigs and additionally arthritis caused by *S. aureus* in three of the 20 kg pigs, whereas none of the 40 kg pigs had developed osteomyelitis or arthritis. Spreading of *S. aureus* to the lungs was seen in two of three 40 kg pigs but only in two of seven 20 kg pigs. As most of the 20 kg pigs were given penicillin, we cannot know whether the favorable results in the 20 kg pig-group were purely related to their body weight and younger age, or whether the pattern of infection also was modified by penicillin treatment. However, we are convinced that the health status and success rate can be improved by using 20 kg pigs treated with penicillin.

Introduction

S. aureus is the primary cause of osteomyelitis in humans (*Berbari et al., 2005; Carek et al., 2001*). In children, most cases of osteomyelitis are caused by hematogenous *S. aureus* infections in growth zones of long bones. Several animal models of *S. aureus* osteomyelitis exist, including models in rabbits, rats, mice, sheep, dogs, goats, pigs, guinea pigs and hamsters (*Reizner et al., 2014*). Rabbits are the most frequently used species in osteomyelitis models, as they are non-expensive and with bones large enough for most procedures. However, in scanning studies – such as PET and SPECT - large animals are preferred due to the low spatial resolution of the scanners and the need for repeated blood sampling. Sheep are commonly used models in osteomyelitis research; however, for testing the usefulness of oral antibiotics, monogastric species such as pigs are preferable (*Johansen & Jensen, 2013*). Furthermore, we have experience in keeping pigs anaesthetized for many hours during PET scans. Recently, we co-authored articles describing the development and characterization of an animal model of juvenile osteomyelitis produced by inoculation of *S. aureus* into the right femoral artery of young domestic pigs (*Johansen et al., 2012; 2013*). The pigs developed foci of osteomyelitis in the ipsilateral limbs, with insignificant signs of further spread to internal organs. Later, we used this model for comparing PET and SPECT radiotracers that are used for diagnosing osteomyelitis (*Nielsen et al., 2015*). However, we chose to modify the original setup by conducting our scan studies in pigs with a body weight of 40 kg instead of the approximately 25 kg which were used in the original model. This change was done in order to be able to perform repeated blood sampling during several dynamic PET scans, and thus to be able to establish blood time-activity curves and analyze tracer metabolites in plasma. With a series of eight pigs, we found the model to be useful in comparing SPECT and PET radiotracers seven days after inoculation; though critically, we had to euthanize three of the eight (38 %) pigs before scanning could be performed due to predefined humane endpoints, which included clinical signs of spread of infection to the lungs. One of four scanned pigs (25 %) failed to develop osteomyelitis during the seven days (*Nielsen et al., 2015*). As the spreading of *S. aureus,* which is also very problematic from an animal welfare viewpoint*,* could explain the model problems we decided to refine the model before using it in further scan studies.

Materials and methods

We studied ten female domestic pigs (Danish Landrace x Yorkshire crossbreds) based on a license provided by The Danish Experimental Animal Inspectorate (license number 2012-15-2934-00123). The pigs were born in a SPF facility and were raised in the same herd. Prior to the study, clinical examination and an automated complete blood cell count (ADVIA 120 Analyzer, Bayer Healthcare Diagnostics, Berlin, Germany), including a leukocyte differential count, using EDTA-stabilized whole blood, were carried out to indicate that all pigs were healthy. We inoculated the pigs with the S54F9 strain of *S. aureus* (8,000-30,000 CFU/kg – grown on bouillon), isolated from a chronic embolic pulmonary porcine abscess, using a right femoral artery access as previous described (*Johansen et al., 2012; 2013*). Three of the pigs had a body weight of 40 kg (± 1 kg), while the remaining seven pigs had a body weight of 20 kg (± 1 kg) at inoculation. If the pigs showed anorexia for more than 24 hours, shallow respiration or fever, the pigs were euthanized, a decision based on the humane endpoints defined in the animal license. The pain (and degree of lameness) was scored once (morning) or twice a day (morning and afternoon) by visual inspection of hind limbs. The scoring includes three steps: **A**: Is the pig standing/walking (yes/no)? **B**: When standing/walking, is the pig using right limb (yes/no)? **C**: Is the pig relieving right hind limb by adding weight to the other three legs or is there any redness, swelling or pain on palpation (yes/no)? All decisions about treatments and euthanasia were taken by a veterinarian: For **A**: if *no* then the pig was killed. For **B**: if *no* then the pig was treated with painkillers and penicillin, and if that was not enough within 24 hours then the pig was killed. For **C**: if *yes*, the pig was treated with painkiller. Pain was scored three times per day (morning, afternoon and evening) after starting giving painkiller. Furthermore, we treated seven of the ten pigs (40 kg; n=1, and 20 kg; n=6) with 10,000 IE/kg procaine benzylpenicillin (Penovet, Boehringer Ingelheim, Copenhagen, Denmark) IM once daily for five days from the onset of lameness (when *no* in **B**) or anorexia. The lameness of a single 20 kg pig deteriorated so rapidly that it did not get antibiotic treatment before euthanasia. After two weeks, the remaining pigs were euthanized and all pigs were kept frozen at -18 °C until necropsy which was performed according to Madsen and Jensen (2011). The main focus of the necropsy was identification of osteomyelitis, any other lesions in right hind limbs, and spread to lungs and other inter-

nal organs. Predefined tissues (lungs, right stifle and tarsal joints) and tissues with macroscopic lesions (arthritis and osteomyelitis) were sampled for microbial cultivation as described by Nielsen *et al.* (2015). The samples were plated on 5% HBA and read after incubation overnight. *S. aureus* was confirmed by latex agglutination (Monostaph Plus, Bionor Laboratories, Norway). Growth characteristics, including a pan-susceptible antibiogram, were taken to indicate identity of the inoculated strain.

Results

The results are shown in Table 1. Lameness and pain in the right hind limb were observed on day three after inoculation in one of the three 40 kg pigs (33 %) and between one and five days after inoculation in all seven 20 kg pigs (100 %). One of the three 40 kg pigs (33 %) and five of the seven 20 kg pigs (71 %) were euthanized before day 14; this took place due to fever and shallow respiration in the 40 kg pig and due to lameness and pain in hind limbs in 20 kg pigs. Necropsies of the three 40 kg pigs showed that none developed osteomyelitis, while six of the seven 20 kg pigs (86 %) developed osteomyelitis caused by *S. aureus*. Three of the 20 kg pigs (43 %) developed arthritis caused by *S. aureus* (and a single (14 %) developed sterile arthritis), whereas none of the 40 kg did. In two of the three 40 kg pigs (67 %) *S. aureus* had spread to the lungs, while only two of the

seven 20 kg pigs (28 %) had this lung infection. All six penicillin-treated 20 kg pigs (100 %) developed osteomyelitis.

Discussion

Our results suggest that intra-arterial inoculation with *S. aureus* in 20 kg pigs and treatment with procaine benzylpenicillin, a long-acting prodrug, increased the success rate for developing osteomyelitis. In addition, we reduced the risk of spread of infection to the lungs. It is not entirely clear how (or if) the younger age and lower body weight of pigs *per se* may prevent localization of the infection to the lungs. In pigs, the immune system matures within the first 10-15 weeks of age (*Juul-Madsen et al., 2010*), which theoretically should favor the 40 kg pigs as the most resistant. In children, risk factors are poorly elucidated, except for distinct incidence peaks in infancy as well at school age (*Blyth et al., 2001; Riise at al., 2008*). We found a good correlation between clinical observations and necropsy findings, as all pigs with osteomyelitis developed lameness and pain in the right hind limb. One 20 kg pig developed clinical signs of lameness and pain without osteomyelitis, which may be explained by arthritis caused by *S. aureus* found in this pig. All three pigs with *S. aureus* arthritis were euthanized on day 3-9 due to lameness and pain in right hind limbs, while only two of the four pigs that were affected solely by osteo-

Table 1. Body weight, treatments, time of first clinical signs, and euthanasia, lesions and bacteriology in ten domestic pigs.

Body weight	Treatment with penicillin§	Day of first clinical hind limb signs, i.e. lameness and pain on palpation	Day of euthanasia	*S. aureus* osteomyelitis	*S. aureus* spread to lungs	*S. aureus* spread to joints
40 kg	-	-*	14	-	-	-
	-	-**	14	-	+	-
	+	3	5[S]	-	+	-
20 kg	-	1	3[L]	-	-	+[K,F]
	+	5	14	+	-	-
	+	3	9[L]	+	-	+[K]
	+	4	14	+	-	-
	+	4	7[L]	+	-	+[T]
	+	4	6[L]	+	+	-
	+	3	4[L]	+	+	-***

§: 10,000 IE/kg procaine benzylpenicillin intramuscularly once daily.

*: No clinical hind limb signs, but transient fever on day 5.

**: *S. aureus* spread to the brain, but without clinical signs.

***: Sterile arthritis in right stifle (knee) joint.

S: cause of euthanasia was body temperature > 40 ˚C (fever) and clinical signs of pneumonia.

L: cause of euthanasia was lameness and other clinical signs of pain in right hind limb.

K: Stifle joint, right.

F: Femuro-tibial and femuro-patellar joints, right

T: Talocrural joint, right.

myelitis were euthanized before the end of the study period (day 14). Of these two, one had sterile arthritis. Therefore, our results indicate that arthritis will add significantly to lameness and pain that result in euthanasia. It may be impossible completely to avoid arthritis when *S. aureus* is injected into the femoral artery. The prevention of spread to the lungs and other organs will, however, reduce suffering and limit the number of pigs used. Additionally, it may also prevent accidental seeding of bacteria to the non-inoculated limb, which serves as a control. As most of the 20 kg pigs were given penicillin, the insignificant spread of bacteria to the lungs probably was an effect both related to the immune function of the pigs combined with a modifying effect by the antibiotic treatment. However, as all six penicillin-treated 20 kg pigs developed osteomyelitis, the chosen formulation and dosage of penicillin did not prevent bone infection. We believe that the lack of osteomyelitis in one of the 20 kg pigs was the result of biological variation. Since treatment with penicillin at onset of first clinical signs did not prevent osteomyelitis and *post mortem* culturing of *S. aureus*, we will, for ethical reasons, not perform further refinement-experiments with 20 kg pigs to determine the separate effect of penicillin treatment and body weight or age. We have not used Non-Steroidal Anti-Inflammatory Drugs (NSAID) in our study, as this could have affected the results by altering immune response to osteomyelitis. However, if additional painkilling in our 20 kg / antibiotics osteomyelitis model proves necessary to comply with the humane endpoints, we will include NSAID to avoid euthanasia and thus the inability to complete our studies.

Conclusion

We conclude that experimentally-induced osteomyelitis and animal welfare can be improved by using 20 kg pigs and antibiotic treatment at onset of the first clinical signs. We do recommend the use of this improved pig model of osteomyelitis, as the risk for spread of infection to lungs is reduced to an acceptable low level. Since we are not completely able to prevent arthritis, we must accept that some pigs will be euthanized due to pain caused by lameness.

References

Berbari EF, JM Steckelberg & DR Osmon: Osteomyelitis. In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and practice of infectious diseases*. Elsevier Churchill Livingstone; 2005: 1322-1332.

Blyth MJ, R Kincaid, MA Craigen & GC Bennet: The changing epidemiology of acute and subacute haematogenous osteomyelitis in children. J. Bone Joint Surg. Br. 2001, *83*(1), 99-102.

Carek PJ, LM Dickerson & JL Sack: Diagnosis and management of osteomyelitis. Am. Fam. Physician. 2001, *63*(12), 2413-2420.

Johansen LK, J Koch, D Frees, B Aalbæk, OL Nielsen, PS Leifsson, TM Iburg, E Svalastoga, LE Buelund, T Bjarnsholt, N Høiby & HE Jensen: Pathology and biofilm formation in a porcine model of staphylococcal osteomyelitis. J. Comp. Pathol. 2012, *147*(2-3), 343-353.

Johansen LK, EL Svalastoga, D Frees, B Aalbæk, J Koch, TM Iburg, OL Nielsen, PS Leifsson & HE Jensen: A new technique for modeling of hematogenous osteomyelitis in pigs: inoculation into femoral artery. J. Invest. Surg. 2013, *26*(3), 149-153.

Johansen LK & HE Jensen: Animal models of hematogenous *Staphylococcus aureus* osteomyelitis in long bones: a review. Orthoped. Res. Rev. 2013, *5*, 51-64.

Juul-Madsen HR, KH Jensen, J Nielsen & BM Damgaard: Ontogeny and characterization of blood leukocyte subsets and serum proteins in piglets before and after weaning. Vet. Immunol. Immunopath. 2010, *133*, 95-108.

Madsen LW & HE Jensen: Necropsy of the Pig. In: Jensen HE, editor. *Necropsy. A handbook and Atlas*. 83-106. Biofolia, 2011.

Nielsen OL, P Afzelius, D Bender, HC Schønheyder, PS Leifsson, KM Nielsen, JO Larsen, SB Jensen & AKO Alstrup: Comparison of 111In-leukocyte single-photon emission computed tomography (SPECT) and Positron Emission Tomography (PET) with four different tracers to diagnose osteomyelitis in a juvenile porcine experimental haematogenous Staphylococcus aureus model. *Am. J. Nucl. Med. Mol. Imagn. 2015, 15(2), 169-182.*

Reizner W, JG Hunter, NT O´Malley, RG Southgate, EM Schwarz & SL Kates: A systematic review of animal models for staphylococcus aureus osteomyelitis. Eur. Cells Mat. 2014, *27*, 196-212.

Riise ØR, E Kirkhus, KS Handeland, B Flatø, T Reiseter, M Cvancarova, B Nakstad & KO Wathne: Childhood osteomyelitis-incidence and differentiation from other acute onset musculoskeletal features in a population-based study. BMC Pediatr. 2008, *8*(45). doi: 10.1186/1471-2431-8-45.