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A porcine model of acute *Staphylococcus aureus* sepsis and pyemia

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Running head: *S. aureus* sepsis model in pigs

25 SUMMARY

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29 pyemia

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31 Sepsis caused by *Staphylococcus aureus* constitutes an important cause of morbidity and mortality
32 in humans, and the incidence of this disease-entity is increasing. In this paper we describe a porcine
33 model of acute, *S. aureus* induced sepsis and pyemia. The study was based on examination of blood
34 samples from anaesthetized and intravenously inoculated pigs, followed by euthanasia and
35 necropsy, either four, five or six hours after inoculation. Clearance of bacteria from the blood was
36 completed within the first two hours in some of the pigs, and the highest bacterial load was recorded
37 in the lungs as compared to the spleen, liver and bones. This probably was a consequence of both
38 the intravenous route of inoculation and the presence of pulmonary intravascular macrophages.
39 Inoculation of bacteria induced formation of acute microabscesses in the lungs, spleen and liver, but
40 not in the kidneys or bones. A generalized inflammatory response reflected in the blood, i.e.
41 leukocytosis and increased levels of IL-6 and CRP, was not recorded, probably due to the short time
42 course of the study. The model forms a basis for future studies of the initial pathogenesis of sepsis
43 and pyemia in the pig, modelling these syndromes in man.

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45 Keywords: Sepsis; model; *Staphylococcus aureus*; pigs; swine; porcine

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71 INTRODUCTION

72 Sepsis constitutes an important cause of morbidity and mortality in humans, and the incidence of
73 this disease-entity is increasing. At present, 660,000 cases of sepsis occur in the USA each year and
74 combined with the high mortality, this ranks sepsis as a leading cause of death in this country.

75 Staphylococci, including methicillin resistant *Staphylococcus aureus* (MRSA), have become the
76 most frequently isolated bacteria in nosocomial infections, giving rise to more than 50% of the
77 cases (1). Similar observations have been made in other countries, including Denmark (2,3).

78 Staphylococcal seeding to e.g. endocardium, skeleton and lungs, resulting in the development of
79 pyemic lesions (infective endocarditis, pyogenic osteomyelitis and lung abscesses) are serious
80 complications to sepsis (4-6).

81

82 Models of bacteraemia, sepsis and pyemia caused by *S. aureus* have been established primarily in
83 small laboratory animals (mice, rats, guinea pigs, and rabbits), and studies of experimental blood
84 stream infection with *S. aureus* in pigs are few. Some of these studies in pigs were aimed at the
85 characterization of pulmonary intravascular macrophages such as the study by Winkler (7), some
86 were models of prosthetic device infections exemplified by that reported by Paget et al. (8), and
87 some investigated the pulmonary haemodynamics and function such as those by Walther et al.
88 (9,10). A few, extensive, studies modelling the pathogenesis of human sepsis and the ensuing shock
89 have been performed with group A streptococci in pigs (11-14), but most of this type of research
90 has been performed with Gram-negative bacteria or endotoxin (15).

91

92 Pigs spontaneously can develop pyemia, and based on records from the post mortem meat
93 inspection, approximately 125,000 pigs (0.4% of the total number of slaughtered pigs) are each year
94 diagnosed with pyemia in Denmark (Ministry of Food, Agriculture and Fisheries, Danish

95 Veterinary and Food Administration, unpublished data 2007). In a study of pyemic lung lesions in
96 pigs, *S. aureus* was found in monoculture in 46 % of the cases (16). Pig farming has been found to
97 be a risk factor for increased nasal *S. aureus* colonization in man, and sequence typing and
98 phylogenetic comparisons of isolates have suggested a high rate of strain exchange between pigs
99 and pig farmers (17). Similar studies on MRSA showed an increased prevalence rate of nasal
100 colonization in persons in contact with pigs (18), and infections in humans with MRSA have been
101 related to a domestic animal source that included pigs (19,20).

102

103 The aim of the present study was to develop and validate a *S. aureus* bacteraemia, sepsis, and
104 pyemia model in pigs, with special focus on the initial lesions and pathophysiology. Such a model
105 has not previously been established. With the increasing use of the pig in biomedical research, this
106 model could prove useful to the study of the disease in man, but also the disease in pigs, as
107 spontaneous generalized *S. aureus* infections is a major concern in both species.

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119 MATERIALS AND METHODS

120 *Animals and housing*

121 Nine clinically healthy Yorkshire-Landrace-Duroc crossbreed pigs (nos. 1-9, corresponding to the
122 journal nos. 36554-36562), body weight of 20-25 kg corresponding to 9-10 weeks of age, were
123 purchased from a commercial pig herd certified free from infections with *Mycoplasma*
124 *hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Brachyspira hyodysenteriae*, toxin producing
125 *Pasteurella multocida*, porcine respiratory and reproduction syndrome (PRRS) virus, *Sarcoptes*
126 *scabiei* var. *suis*, and *Haematopinus suis*. Furthermore, the herd was considered free from a range of
127 other infections according to the national animal health status of Denmark (Ministry of Food,
128 Agriculture and Fisheries, Danish Veterinary and Food Administration
129 [http://www.foedevarestyrelsen.dk/Dyresundhed/Dyresygdomme_og_zoonoser/Sygdomsoversigt/fo
130 [rside.htm](http://www.foedevarestyrelsen.dk/Dyresundhed/Dyresygdomme_og_zoonoser/Sygdomsoversigt/fo)]).

131

132 The pigs were allowed to acclimatize for 5-10 days before entering the trial. Food was withdrawn
133 12 h before the start of the experiment, and immediately before the start the pigs underwent a
134 clinical examination and measurement of body temperature, to secure absence of clinical signs of
135 disease.

136

137 *Experimental design*

138 The pigs were sedated by intramuscular injection of a solution containing a mixture of zolazepam,
139 tiletamine, xylazine and ketamine (0.83 mg/kg body weight of each of the drugs), and butorphanol
140 (0.17 mg/kg body weight). A catheter (22 G) was then inserted in the right ear vein and used for
141 infusion of anaesthetics, which consisted of a solution containing a mixture of xylazine (1 mg/ml),
142 ketamine (2 mg/ml), butorphanol (0.1 mg/ml) and guaifenesine (48 mg/ml). Anaesthesia was

143 monitored by the ciliar and corneal reflexes. Also, the heart rate was continuously monitored and
144 rectal temperature was measured at regular intervals. After the induction of anaesthesia the pigs
145 were positioned in dorsal recumbency, and a tracheal tube was applied and used for artificial
146 ventilation at a volume of 200 ml and a rate of 14 per min if signs of cardiovascular and respiratory
147 incompetence (ruffled hair coat, oedema of the eyelids, erythema of the skin and respiratory arrest)
148 were observed. This prompted artificial ventilation in all pigs except nos. 2 and 7.

149

150 A catheter (22 G) was inserted in the left ear vein and used for the administration of bacteria or
151 mock followed by flushing with 10 ml sterile isotonic saline. After this procedure the catheter was
152 removed. Another catheter (diameter of 2.6 mm) was surgically inserted into the left external
153 jugular vein, adjusted to sit in the bi-jugular trunk, fixed to the skin with stitches, and flushed with
154 10 ml sterile isotonic saline followed by 2 ml of sterile 18 EI heparin solution. This catheter was
155 used for blood sampling, and the samples were secured free of heparin solution by discarding the
156 first 5 ml of blood. Blood from pig no. 2 was not drawn from the catheter, but taken directly, with a
157 hypodermic needle, from the bi-jugular truncus or left external jugular vein. All surgical
158 procedures, insertion of catheters, injections, withdrawal of blood, aliquotation of blood etc. was
159 performed strictly aseptically using 70% ethanol as disinfectant.

160

161 Eight pigs (nos. 1-6, 8 and 9) were inoculated with *S. aureus*, and one (no. 7) was mock-inoculated
162 with sterile isotonic saline. Examination of blood included bacteriology and haematology on full
163 blood, IL-6 on plasma and C-reactive protein on serum. The blood samples were taken regularly
164 until killing of the pigs four hours after inoculation (PI) (nos. 1-4 and 7), five hours PI (nos. 6 and
165 9) or six hours PI (nos. 5 and 8), and included pre-inoculation blood samples from the anaesthetized
166 pigs (Table 1). The post mortem examination included bacterial culture from organs,

167 histopathology, and fluorescent *in situ* hybridisation for bacteria. The study was conducted in
168 accordance with the EU directive 86/609 and the Danish Animal Experimentation Act.

169

170 *Staphylococcus aureus* suspension

171 *Staphylococcus aureus*, isolate no. S54F9 was obtained from a chronic embolic pulmonary abscess
172 in a Danish slaughter pig (Department of Veterinary Pathobiology journal number 36444). The
173 isolate was identified using Api ID 32 Staph (Biomérieux, Inc., Marcy-l’Etoile, France) and was
174 propagated in 100 ml of Luria-Bertani (LB) broth (21) for 18 h at 37°C, sedimented by
175 centrifugation and re-suspended in sterile isotonic saline. The viable count was determined by
176 counting the number of colonies formed on LB agar medium inoculated with 10 µl volumes of ten
177 fold dilutions and incubated at 37°C for 24 h. The suspension was diluted with sterile isotonic saline
178 to obtain a suspension containing 10⁸ colony-forming units (CFU) per ml. This was used for
179 intravenous inoculation at a dose of 10⁸ CFU (1 ml) per kg of body weight.

180

181 *Bacteriological examination of blood and organs*

182 Heparin-stabilized blood samples of 10 ml were taken aseptically as indicated (Table 1). The blood
183 samples were kept at 5°C for a maximum of 4 h until being processed. The blood samples in
184 volumes of 1 ml and 1 ml of decimal dilutions (using sterile isotonic saline) were added to empty
185 Petri dishes and mixed with melted LB agar medium. Viable count was determined after incubation
186 for 48 h at 37°C , and presented as counts per ml of blood.

187

188 Quantitative bacteriological examination was performed on the lung (left diaphragmatic lobe),
189 spleen (dorsal half) and liver (left lateral lobe) from all nine pigs upon euthanasia. In addition, bone

190 tissue from the metaphysis/physis region of the left femur was cultured in four of the pigs (nos. 5, 6,
191 8, 9). The samples were kept at 5°C for a maximum of 12 h before being processed.

192

193 Approximately 1 g of tissue was removed aseptically from the organs, cut into minor pieces with a
194 scalpel, weighed and homogenized in 9 ml of sterile isotonic saline using a stomacher. Ten fold
195 dilutions in sterile isotonic saline of the homogenized tissues were prepared. From each of these 10
196 µl were inoculated on the surface of an LB agar medium and incubated for 48 h at 37°C before
197 counting the colonies. The counts per g of tissue were calculated. Colony morphology was
198 evaluated and representative colonies were subcultured on blood agar (Blood agar base, CM55;
199 Oxoid, Basingstoke, Hampshire, England) containing 5% sterile bovine blood and phenotypically
200 characterized using Api ID 32 Staph.

201

202 *Haematology*

203 Blood samples (Table 1) for haematology were stabilized with EDTA and total and differential
204 white blood cell counts, and counts of platelets, were performed. The blood samples were kept at
205 5°C for up to 24 h before processing. The ADVIA 120 (Bayer) automated haematology analyser
206 with a species specific software setting was used. The analyser is subjected to internal and external
207 quality control and only results from accepted runs were released. Blood smears were evaluated as a
208 secondary check for the automated analysis (22).

209

210 *Assays for plasma IL-6 and serum C-reactive protein*

211 Plasma was generated by centrifugation of EDTA stabilised blood sampled in endotoxin free vials.
212 Centrifugation was performed immediately after blood had been collected (Table 1). The plasma
213 samples were kept at 5°C for maximum one h before storing at - 80°C. The IL-6 content was

214 determined in plasma by an R & D Systems DuoSet ELISA (R & D Systems, Abingdon, UK,
215 catalog no. DY686), using ELISA plates from Nunc (Roskilde, Denmark, type: Macrosorp), and
216 using goat anti porcine IL-6 for coating (0.8 µg/ml in PBS) and biotinylated goat anti porcine IL-6
217 for detection (0.1 µg/ml in PBS with 1% bovine serum albumin (BSA), Sigma St. Louis, MO,
218 catalog no. A2153). Coating was 100 µl pr well overnight at room temperature, followed by 3 times
219 washing in PBS containing 0.05% Tween 20. All subsequent washings were done in the same way.
220 Then plates were blocked for 1 h in PBS containing 1% BSA, 300 µl pr. well, followed by washing
221 and incubation of samples, diluted twice in 100 µl PBS with 1% BSA. All subsequent incubations
222 were done in 100 µl. A standard preparation of recombinant porcine IL-6 (from the DuoSet kit) was
223 applied in double determination as a two-fold dilution row from 8000 pg/ml to 125 pg/ml. Two
224 wells were used for buffer controls. This was incubated for 2 h at room temperature, washed and
225 incubated with detection antibody for 2 h. After washing, the plate was incubated for 20 min at
226 room with peroxidase-conjugated streptavidin (from the DuoSet kit, diluted 1/200) followed by
227 washing. Plates were developed with TMB Plus from Kem-En-Tec (Taastrup, Denmark) incubating
228 10-20 min at room temperature in the dark and then stopped by the addition of 100 µl 0.5 M
229 sulfuric acid pr. well. Plates were read in a Thermo Multiskan Ex spectrophotometer (Thermo
230 Scientific, Waltham, MA) at 450 nm subtracting background (540 nm). Sample values for IL-6
231 were then calculated from the curve fitted to the readings of the standard (using Ascent software v.
232 2.6).

233

234 Serum was generated by centrifugation of blood samples (Table 1) left to coagulate for no longer
235 than one hour at 22°C in plain, endotoxin free vials. The serum samples were kept at 5°C for
236 maximum one hour before storing at - 80°C. The C-reactive protein (CRP) content in serum was
237 determined on the ADVIA 1650 (Bayer) as previously described (23).

238

239 *Post mortem examination and histopathology*

240 The post mortem examination included median or sagittal sections of the bones. Tissue samples of
241 the lungs (dorsal part of the diaphragmatic lobes), the spleen, the liver, the kidneys, and the
242 metaphysis/physis region of the right femur, tibia, radius, ulna, sacral bone, thoracic vertebrae 8 and
243 9 plus the costochondral junction of the 8th and 9th ribs were fixed for 24 h in PBS buffered 4%
244 formaldehyde. After fixation bone tissues were decalcified for six days in a solution of
245 ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide (280 g EDTA and 30 g NaOH
246 dissolved in 2000 ml of water). The tissue specimens were then processed through graded
247 concentrations of ethanol and xylene, embedded in paraffin wax, cut at 3-5 µm, rehydrated, and
248 stained with haematoxylin and eosin (HE) (24). Tissue sections for *in situ* hybridisation were
249 mounted on Super Frost Plus glass slides (Gerhard Menzel, Braunschweig, Germany), and
250 processed as stated below.

251

252 *Fluorescent in situ hybridisation*

253 Fluorescent *in situ* hybridisation (FISH) was applied on selected tissue sections using an Alexa 555
254 5'-labeled oligonucleotide probe (EUB 338) targeting a 16S rRNA sequence specific for the
255 Domain Bacteria (25). The procedure was modified after Boye et al. (26), and included a 10 min
256 pre-treatment at 20°C of the sections with 3 mg/ml of lysozyme (cat. no. L-6876, Sigma Aldrich,
257 USA) dissolved in a Tris/EDTA-buffer (100mM Tris and 50 Mm EDTA (pH 6.5)). The sections
258 were rinsed in water and hybridised for 16 h in a moist chamber at 40°C with 5ng/ml of probe
259 dissolved in a hybridisation buffer (700 mM NaCl, 100 mM Tris (pH 8) and 0.1% sodium dodecyl
260 sulphate (SDS)). Washing of the sections was performed 2 times with 2 X standard saline citrate

261 (SSC) for 1 min, with hybridisation buffer prewarmed to 45°C for 20 min, and finally 2 times with

262 2 X SSC for 1 min.

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282 RESULTS

283 *Diseases unrelated to the experimental infection (background pathology)*

284 While performing the post mortem examination some lesions were noted that could be assigned to
285 agents and factors unrelated to the experimental *S. aureus* infection. Thus two pigs (nos. 1 and 2)
286 were diagnosed with subacute to chronic pneumonia affecting the cranial part of the right lung.
287 Histopathology confirmed the presence of chronic purulent bronchopneumonia in pig no. 1 and
288 chronic bronchiointerstitial pneumonia in pig no. 2. Two pigs (nos. 4 and 6) showed macroscopic
289 lesions in the ileum consistent with proliferative ileitis. Samples of ileum from all nine pigs were
290 then examined by immunohistochemistry (27) for the combined presence of histological lesions and
291 the causative agent *Lawsonia intracellularis*. Thus all pigs, except nos. 5 and 7 were diagnosed with
292 the infection. Finally, several of the pigs had histological signs of chronic multifocal interstitial
293 nephritis.

294

295 *Bacteriological examination of blood and organs*

296 The viable counts obtained from blood and tissue samples are given in Table 2. All
297 colonies had a morphology identical to that of the inoculation strain. Representative isolates showed
298 the same reaction pattern in API Staph as the strain used for inoculation of the pigs.

299

300 *Haematology*

301 Pig no. 4 and 9 (*S. aureus*-inoculated group) showed neutrophilia, when compared to normal levels
302 ($3.1 - 11.2 \times 10^9/l$) of neutrophils (28). In pig no. 4, neutrophilia ($12.5 - 17.4 \times 10^9/l$) was seen
303 throughout the experiment, except for the last sample taken 240 min PI, and thus also in the blood
304 sample taken before inoculation of bacteria. Pig no. 9 had marginally increased levels of neutrophils
305 ($11.6-12.0 \times 10^9/l$) at 270 and 300 min. The two pigs showed no changes in lymphocyte, monocyte,

306 eosinophil, basophil or platelet numbers, and no changes at all were found in the remaining seven
307 pigs, including the mock-inoculated.

308

309 *Plasma IL-6 and serum C-reactive protein*

310 On each of the time points tested, the content of IL-6 was under the detection limit of the assay
311 which was 250 pg/ml. Also, CRP measurements did not reveal any significant increases or
312 variations, except for to aberrant measures in two pigs most likely resulting from technical errors.

313

314 *Gross pathology , histopathology and in situ hybridisation*

315 Gross lesions related to the experimental design were atelectasis of the dorsal part of the
316 diaphragmatic lobes. This was seen in all pigs, and was probably related to their dorsal recumbency
317 during anaesthesia.

318

319 The HE stained section of the lungs revealed presence of acute microabscesses (Fig. 1) and
320 aggregates of spherical, basophilic organisms in three of the four *S. aureus*-inoculated pigs killed 5
321 or 6 h PI (nos. 5, 6, 8). These aggregates were identified as bacterial colonies by *in situ*
322 hybridisation with a general bacterial oligonucleotide probe, and bacterial colonies were often
323 present without any ensuing inflammatory reaction (Fig. 2). All *S. aureus*-inoculated pigs killed 4 h
324 PI, one pig killed 5 h PI and the mock-inoculated pig were without histological lung lesions.

325

326 In the spleen of all *S. aureus*-inoculated animals killed 5 or 6 h PI (nos. 5, 6, 8, 9), acute
327 microabscesses were present in the marginal zone, the zone between the red and white pulpa. In two
328 of the *S. aureus*-inoculated pigs killed 4 h PI (nos. 1, 3) neutrophils seemed to accumulate in the
329 marginal zone without forming true microabscesses. The remaining pigs (two *S. aureus*-inoculated

330 and the mock-inoculated) were without histological lesions. In the liver, acute microabscesses were
331 detected in pigs from the staphylococcus group killed 4 h PI (nos. 1, 2, 4), 5 h PI (nos. 6, 9), and 6 h
332 PI (no. 8). The rest of the pigs, including the mock-inoculated, were without histological lesions.
333 The kidneys showed no *S. aureus* related histological lesions. The metaphyses of all bones from
334 both inoculated and control animals were without histological lesions.

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352 DISCUSSION

353 The quantitative bacteriological examination of blood and tissues (Table 2) showed that blood
354 samples taken 2 min after intravenous injection of *S. aureus* contained an initial mean viable count
355 of 1800 CFU/ml. The subsequent blood samples showed decreasing numbers of bacteria reaching a
356 mean of 2 CFU/ml 4 h PI, and with three animals being culture negative. These results reflect both
357 dilution of the inoculated bacteria within the blood compartment and clearance.

358

359 In the organs, which were examined 4, 5 or 6 h PI, mean viable counts largely exceeded the initial
360 viable count obtained in the blood, indicating a considerable capacity of the organs for withholding
361 bacteria from the circulation. The mean viable count per g of spleen and liver tissue were of the
362 same magnitude, 24,000 CFU/g and 21,000 CFU/g, respectively, in contrast to a higher mean viable
363 count from lung tissue, 110,000 CFU/g, suggesting that the porcine lung has a high capacity for
364 retaining bacteria from the circulation. This finding is in agreement with previous reports (29,30),
365 and is closely linked to the clearing action of pulmonary intravascular macrophages (PIM), present
366 in swine and many other animal species, but not in man (7). When comparing the content of
367 bacteria in different organs, the volume of blood and the blood-load of bacteria entering the organs
368 should be taken into account (30). Thus, the lungs will receive the total volume of blood and load of
369 bacteria, whereas the spleen, for example, will only receive a fraction of the blood and only bacteria
370 that is not withheld by the lungs or other organs. Also, proliferation or destruction of the bacteria
371 within the organs would influence the level. Thus destruction of bacteria in the lungs could explain
372 the seemingly lower levels in the lungs of pigs examined 5 and 6 h PI as opposed to pigs examined
373 4 h PI. The mean viable count from the metaphysis/physis region was 2300 CFU/g, the lowest
374 recorded, but, by comparison to the 0 - 2 CFU/ml of blood, still indicates some capacity for the
375 retention of bacteria by bone tissue.

376

377 The *S. aureus* strain used in this experimental infection was originally isolated from a case of
378 embolic apostematous pneumonia in a Danish pig. As part of another study, the strain was typed by
379 tandem repeat analysis of the staphylococcal protein A (*spa*) gene, a standard method for molecular
380 typing of *S. aureus* (31). The *spa* type observed in this strain (t1333) is one of the two most
381 common types among porcine clinical *S. aureus* isolates in Denmark and is associated with clonal
382 complex 30 according to the classification based on multi-locus sequence typing (data not shown).

383

384 Haematology showed neutrophilia in two of the *S. aureus*-inoculated animals. These animals also
385 were infected with *L. intracellularis* which could add to the neutrophilia. Indeed, one of the animals
386 had neutrophilia before inoculation of *S. aureus*, underscoring the effect of pre-inoculation
387 infection, i.e. the observed background pathology of the pigs. Both pneumonia and proliferative
388 ileitis are common diseases among pigs held under industrial conditions. A variety of infectious
389 agents, present in up to 90% of all pig farms, can cause respiratory disease (32). Also, 20-40% of all
390 farms have clinical and/or subclinical cases of proliferative ileitis (33). Chronic multifocal interstitial
391 nephritis does not seem to impair renal function (34) and is probably related to chronic or persistent
392 infections with virus widespread in the pig population (35).

393

394 Whatever the reason was for the observed neutrophilia, plasma IL-6 was not detected in any of the
395 pigs and no increase in serum CRP was observed. IL-6 together with IL-1 and tumor necrosis
396 factor- α (TNF- α) are some of the major proinflammatory cytokines produced in monocytes and
397 other cells as an immediate response to infection and other stimuli. The cytokines have a range of
398 local and systemic effects, including the recruitment of neutrophils and the induction of acute phase
399 proteins from the liver. The systemic effects rely on the presence of cytokines in the blood, and this

400 presence in blood is linked to a range of different factors. For example, endotoxin caused in an *in*
401 *vitro* assay on cells the production of IL-1, IL-6 and TNF α within 1-5 h, whereas Gram-positive
402 toxins induced a peak response of lymphotoxin- α and interferon- γ 50-75 h after challenge (36). In
403 experimental aerogenous infection studies in pigs with the Gram-negative pulmonary pathogen
404 *Actinobacillus pleuropneumoniae*, blood IL-6 was detected within the first 10-14 h after inoculation
405 (37,38), and increase in blood CRP has been demonstrated in several studies (39). The absence of a
406 systemic IL-6 and CRP response in our study could have a variety of explanations, including the
407 short duration of the experiment and the bacterium used. However, IL-6 was detected in the blood
408 only one hour after the intravenous inoculation of *S. aureus* in mice (40), and was produced in
409 response to *in vitro* challenge of human endothelial cells by *S. aureus* (41). Also, TNF- α was
410 detected in blood only three hours after the intravenous inoculation of serogroup A streptococci in
411 pigs (13).

412

413 The histological examination revealed presence of acute microabscesses and bacterial colonies
414 while evidence of thrombosis was absent. Microabscesses were seen in all *S. aureus*-inoculated
415 animals except for one pig (no. 3), and were detected in the lung, spleen and liver, but not in the
416 kidney and the metaphysis/physis of bones. *S. aureus* colonies were present in only the lungs of
417 three *S. aureus*-inoculated pigs. The bacterial colonies could represent trapping of bacterial emboli
418 or local proliferation.

419

420 The presence of microabscesses in the marginal zone of the spleen corresponds well with sepsis
421 (42). Presence of microabscesses in the lungs and the liver probably relates to the presence of PIM
422 in the lungs and Kupffer's cells in the liver (43). Naturally occurring pyemia in pigs is often
423 associated with lesions in the lungs and the skeleton (Ministry of Food, Agriculture and Fisheries,

424 Danish Veterinary and Food Administration, unpublished data 2007). Frequently isolated bacteria
425 are *S. aureus* from lung lesions (16) and *Arcanobacterium pyogenes* from skeleton abscesses (44).
426 *S. aureus* (and other bacteria) also can be isolated from cases of osteomyelitis, and different
427 predisposing factors, including the presence of receptors to bone surface proteins in *S. aureus*, have
428 been offered to explain the frequent occurrence of acute osteomyelitis localized to the metaphysic-
429 or the equivalent epiphysis regions (44). The lack of osteomyelitis in our study could be a result of
430 the short timeframe of the study or the rather light colonization of the skeleton.

431

432 In conclusion we were able to induce the formation of acute microabscesses in pigs examined four
433 to six hours after the intravenous inoculation of *S. aureus*. Microabscesses were present in the
434 lungs, spleens and livers, but not in the kidneys or bones. The presented model thus proved
435 successful for the establishment of acute *S. aureus* associated sepsis and pyemia, and implicates the
436 model as ideal for the future study of the initial pathogenesis of sepsis and pyemia in the pig. A
437 generalized inflammatory response reflected in the blood, i.e. leukocytosis and increased levels of
438 IL-6 and CRP, was, however, not recorded, and future studies should include inoculation with
439 strains of *S. aureus* isolated from man and expansion of the timeframe with the goal of reaching a
440 severe sepsis or septic shock stage. If this can be achieved, the model may prove beneficial from a
441 translational medicine perspective, implicating diagnostics, prognostics and therapy of *S. aureus*
442 sepsis and allied conditions in man.

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472 TABLE 1. Blood sampling
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Pig no.	Inoculation	Test period	Test ¹	Blood sampling timepoints ¹														
				- 2 ²	2 ³	30	60	90	120	150	180	210	240	270	300	330	360	
1-4	<i>S. aureus</i>	4 h	Bacterial culture	+	+	+	+	+	+	+	+	+	+					
			Haematology	+		+	+	+	+	+	+	+	+					
			Plasma IL-6	+		+	+	+	+	+	+	+	+					
			Serum CRP	+		+	+	+	+	+	+	+	+					
5, 8	<i>S. aureus</i>	6 h	Bacterial culture	+	+	+	+	+	+	+	+	+	+				+	
			Haematology	+		+	+	+	+	+	+	+	+	+	+	+	+	
			Plasma IL-6	+		+	+	+	+	+	+	+	+	+	+	+	+	
			Serum CRP	+		+	+	+	+	+	+	+	+	+	+	+	+	
6, 9	<i>S. aureus</i>	5 h	Bacterial culture	+	+	+	+		+				+		+			
			Haematology	+		+	+	+	+	+	+	+	+	+	+			
			Plasma IL-6	+		+	+	+	+	+	+	+	+	+	+	+		
			Serum CRP	+		+	+	+	+	+	+	+	+	+	+	+		
7	Mock	4 h	Bacterial culture	+	+	+	+		+				+					
			Haematology	+		+	+	+	+	+	+	+	+					
			Plasma IL-6	+		+	+	+	+	+	+	+	+	+				
			Serum CRP	+		+	+	+	+	+	+	+	+	+				

474
475 ¹: A few of the test results are missing (not indicated)
476 ²: “-2” indicates that blood was collected 2 min before inoculation
477 ³: Blood was collected 2 min after inoculation (PI)
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486 TABLE 2. Viable count expressed per ml of blood or per g of tissue of *Staphylococcus aureus* in eight pigs following intravenous injection
 487 of 10⁸ bacteria per kg of body weight and one control pig injected with and equal volume of sterile isotonic saline (mock)
 488

Pig no.	Innoculation	Pig killed	Blood sampling timepoints								Organs			
			-2 ¹	2 ²	30	60	120	240	300	360	Lung	Spleen	Liver	Metaphysis/physis region of bone
1	<i>S. aureus</i>	4 h PI	0	2600	29	13	0	0			140000	2800	93000	NT ³
2	<i>S. aureus</i>	4 h PI	0	2200	25	8	5	0			130000	18000	7700	NT
3	<i>S. aureus</i>	4 h PI	0	6500	47	10	2	4			290000	11000	3800	NT
4	<i>S. aureus</i>	4 h PI	0	400	47	11	0	0			110000	2700	9600	NT
5	<i>S. aureus</i>	6 h PI	0	850	50	18	2	1	NT	- ⁴	78000	34000	3300	1400
6	<i>S. aureus</i>	5 h PI	0	1100	100	57	14	4	2		26000	43000	34000	2700
8	<i>S. aureus</i>	6 h PI	0	480	34	12	5	2	NT	0	50000	16000	1200	730
9	<i>S. aureus</i>	5 h PI	0	520	88	46	21	2	1		47000	63000	15000	4300
Mean of 1-6, 8, 9		4 - 6 h PI	0	1800	53	22	6	2			110000	24000	21000	2300
7	Mock	4 h PI	0	0	0	0	0	0	0		0	0	0	NT

489
 490 ¹: “-2” indicates that blood was collected 2 min before inoculation
 491 ²: Blood was collected 2 min after inoculation (PI)
 492 ³: Not tested (NT)
 493 ⁴: Result missing
 494

495

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499 LEGENDS TO FIGURES

500

501 *Fig. 1.* Section of lung from a *S. aureus* infected pig killed 6 h PI (pig no. 5) showing a
502 microabscess located to an alveolar septum. Hematoxylin- and eosin stain. Bar = 50 μm .

503

504 *Fig. 2.* Section of lung from a *S. aureus* infected pig killed 5 h PI (pig no. 6) showing a bacterial
505 colony without any inflammatory reaction and identified by fluorescent *in situ* hybridisation
506 (insert). The *in situ* hybridisation was performed first and the bacterial colony photographed. Then
507 the section was stained with hematoxylin, and the same colony identified and a new photo taken.

508 Bar = 20 μm .

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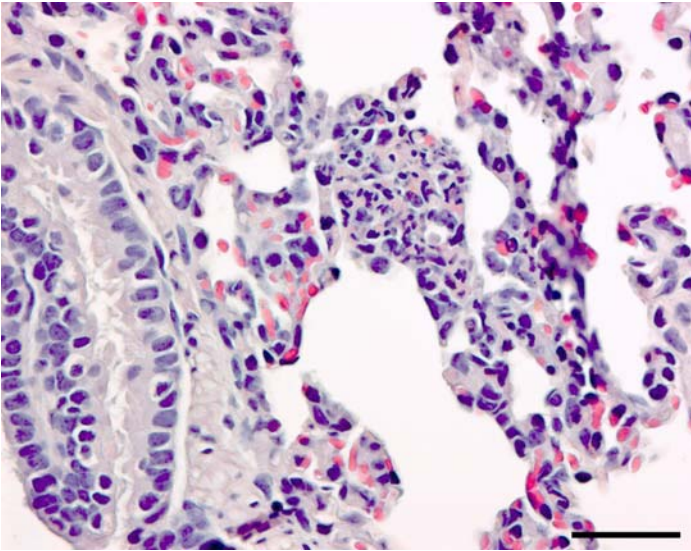
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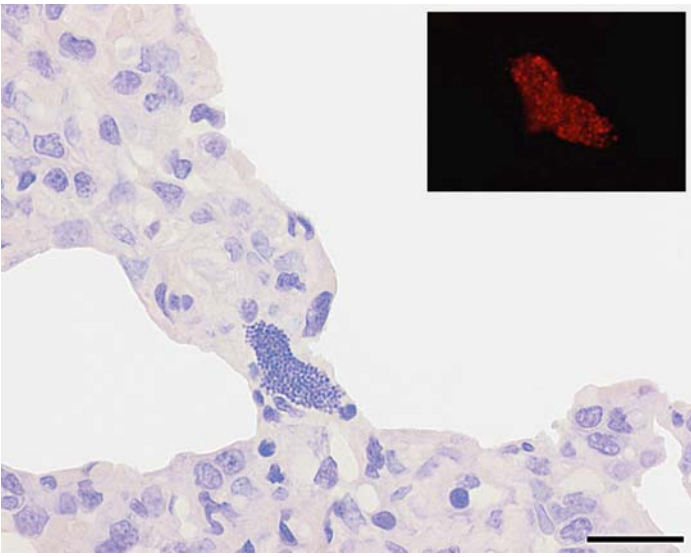
518



519 *Fig. 1*

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522 *Fig. 2*

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