

Porcine Models of Non-Bacterial Thrombotic Endocarditis (NBTE) and Infective Endocarditis (IE) Caused by *Staphylococcus aureus*: A Preliminary Study

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Background and aim of the study: Non-bacterial thrombotic endocarditis (NBTE) and, in particular, infective endocarditis (IE), are serious and potentially life-threatening diseases. An increasingly important agent of human IE is *Staphylococcus aureus*, which typically causes an acute endocarditis with high mortality. The study aim was to evaluate the pig as a model for non-bacterial as well as *S. aureus*-associated endocarditis, as these models would have several advantages compared to other laboratory animal models.

Methods: Fourteen animals underwent surgery with placement of a plastic catheter in the left side of the heart. Six of the pigs did not receive a bacterial inoculation and were used to study the development of NBTE. The remaining eight pigs were inoculated intravenously once or twice with *S. aureus*, 10⁵-10⁷ cfu/kg body weight. Two bacterial strains were used: S54F9 (porcine) and NCTC8325-4 (human). Clinical examination, echocardiography and bacterial blood cultures were used to diagnose and monitor the development of endocarditis. Animals were

ethanized at between two and 15 days after catheter placement, and tissue samples were collected for bacteriology and histopathology.

Results: Pigs inoculated with 10⁷ cfu/kg of *S. aureus* strain S54F9 developed clinical, echocardiographic and pathologic signs of IE. All other pigs, except one, developed NBTE. Serial blood cultures withdrawn after inoculation were positive in animals with IE, and negative in all other animals.

Conclusion: *S. aureus* endocarditis was successfully induced in pigs with an indwelling cardiac catheter after intravenous inoculation of 10⁷ cfu/kg of *S. aureus* strain S54F9. The model simulates typical pathological, clinical and diagnostic features seen in the human disease. Furthermore, NBTE was induced in all but one of the pigs without IE. Thus, the pig model can be used in future studies of the pathogenesis, diagnosis and therapy of NBTE and *S. aureus* endocarditis.

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Endocarditis is a serious disease which is usually classified based on its etiology. Non-bacterial thrombotic endocarditis (NBTE) is characterized by the presence of sterile endocardial vegetations consisting of fibrin, platelets, and other blood components (1,2). Compared to infective endocarditis (IE), the masses in NBTE are usually small, are not associated with significant destruction of cardiac tissue, and inflammatory cell infiltrate is sparse or absent. Nevertheless, they can serve as a source of

embolization to the brain, kidneys and other organs, causing major complications. Blood-borne bacteria can also attach to sterile vegetations and cause IE. NBTE is difficult to diagnose, and the pathogenesis is not fully elucidated. It is associated with a number of different diseases, such as neoplasia (adenocarcinoma, in particular) and disseminated intravascular coagulation. Another well-known predisposing condition is endocardial trauma from indwelling catheters, such as pulmonary artery catheters and central venous catheters (3).

Infective endocarditis is a life-threatening disease characterized by the microbial infection of heart valves or mural endocardium, often with consequent damage of the underlying cardiac tissue. Despite progress in

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Table I: Overview of inoculation strains, dosages, and times of euthanasia.

Group	Pig number	<i>S. aureus</i> strain	Challenge dose (cfu/kg)	Time from catheter placement to euthanasia (days)
A	A-1	NI	NI	2
	A-2	NI	NI	2
B	B-1	NI	NI	4
	B-2	NI	NI	4
C	C-1	NI	NI	5
	C-2	NI	NI	6
D	D-1	S54F9 (porcine)	I ₁ ,10 ⁵	15
	D-2	S54F9 (porcine)	I ₂ ,10 ⁶	15
E	E-1	NCTC8325-4 (human)	I ₁ : 10 ⁵	15
	E-2	NCTC8325-4 (human)	I ₂ ,10 ⁶	15
F	F-1	NCTC8325-4 (human)	I ₁ : 10 ⁷	8
	F-2	NCTC8325-4 (human)		8
G	G-1	S54F9 (porcine)	I ₁ : 10 ⁷	5*
	G-2	S54F9 (porcine)		6*

*Euthanasia due to clinical signs of endocarditis.

I₁: First inoculation at four days after catheter placement, I₂: Second inoculation at 11 days after catheter placement.

NI: Non-inoculated.

the diagnosis and treatment of IE, the mortality remains high (4). *Staphylococcus aureus* is the leading cause of IE, and is associated with an increased mortality compared to other etiological agents, with a one-year mortality rate of approximately 40% (4,5). The increase in methicillin-resistant *S. aureus* (MRSA) infections has been distinct in recent years (6), and these are also important causes of IE (7,8).

Animal models of endocarditis have proved useful for the study of IE, particularly for the invaluable opportunity of evaluating therapeutic efficiency in experimental settings. IE models have been described in a variety of animals (9-12), the most popular being the catheter-induced rabbit model (13-15). However, there are disadvantages in using the rabbit as model, as fundamental differences in the intestinal anatomy and flora between humans and rabbits may cause major limitations in the testing of certain antibiotics (16).

A porcine model of endocarditis has numerous advantages, such as anatomic and physiologic similarities to humans (17). More specifically, there are important comparabilities in key characteristics such as heart size and morphology, coronary blood flow, cardiac output, blood pressure, growth of the cardiovascular system, and platelet adhesive properties (17-19). The size of the pig also makes it

possible to use the same diagnostics as in humans, as well as to study prosthetic valve endocarditis. Pig models for streptococcal endocarditis have been described (20,21), but only one report has described the experimental induction of porcine staphylococcal endocarditis in native heart valves (22). In that study, the bacteria were inoculated subcutaneously. In recent studies where pigs were intravenously inoculated with *S. aureus*, IE was not established, despite a large bacterial inoculum (23,24). Therefore, an alternative approach to model *S. aureus* endocarditis in pigs would be to apply a catheter-induction of NBTE followed by the intravenous inoculation of *S. aureus*. This would mimic a natural infection with *S. aureus* bacteremia, as seen frequently in humans (25).

Thus, the study aim was to evaluate the pig as a predictable and reproducible model for non-bacterial as well as *S. aureus*-associated endocarditis.

Materials and methods

Animals and housing

Fourteen clinically healthy female Yorkshire-Landrace cross-breed pigs (age 6-9 weeks; body weight 13-19 kg) were used in this study. The animals originated from a Specific Pathogen-Free (26) herd. On arrival, the pigs were allocated at random to

experimental groups and housed pairwise in separate pens. They were fed a conventional porcine diet (NAG Svinefoder 5, Helsingør, Denmark) and had free access to tap water.

Experimental protocol

After an acclimatization period of seven days, all animals underwent surgery with placement of a plastic catheter in the left side of the heart. As shown in Table I, six pigs (Groups A-C) did not receive a bacterial inoculation, and were euthanized on postoperative days 2, 4, 5, and 6, in order to examine the development of sterile thrombotic lesions. Eight animals (Groups D-G) were bacterially inoculated on day 4 after catheter placement, and challenged with either the porcine *S. aureus* strain S54F9 (24,27) or the *S. aureus* strain NCTC8325-4, derived from a human clinical sepsis isolate (8325), in dosages as described in Table I. As no sign of IE was present in pigs of Groups D and E on day 7 post-inoculation (PI), a second bacterial inoculation was given. Animals were euthanized on days 2-15 after surgery (Table I).

During the study, all animals were monitored clinically and ultrasonographically for signs of endocarditis, and blood samples for bacterial cultivation were withdrawn at regular intervals. The procedures were performed in accordance with the Danish National Guidelines for Animal Care and were approved of by The National Authority (The Animal Experiments Inspectorate) in Denmark (license No. 2008/561-37).

Surgical procedure, inoculation and euthanasia

Before surgery, the animals were sedated as described previously (23), and anesthesia was induced and maintained with propofol (Propofol plus, 10 mg/ml given at 1 ml/kg/h; Orion Pharma, Nivå, Denmark). Animals were placed in dorsal recumbency with both front limbs drawn caudally to facilitate catheter insertion. The surgical procedure was performed aseptically. An incision was made in the ventral midline of the neck, the right carotid artery was localized and freed from the surrounding tissue, and two ligatures (Vicryl 4/0 Ethicon; Jonson & Jonson Company, St. Stevens Woluwe, Belgium) were placed around it. Cranially, the ligature was tightened. The caudal ligature remained loose, an artery clamp was placed caudal to it, and a small opening was cut in the isolated artery segment. The catheter (06/08 PVC phthalate-free tube with X-ray line; Unomedical Inc., McAllen, USA), was inserted into the artery in the direction towards the heart. Prior to insertion, the distal end of the catheter was scraped lightly with a pair of scissors to roughen the surface; in a pilot study (unpublished data) this promoted endocardial lesions.

Furthermore, the catheter end was cut in an oblique direction to generate a pointed shape to facilitate catheter insertion. When loosening the artery clamp the catheter was advanced with ultrasonographic guidance approximately 18 cm to fit into the left cardiac ventricle. Thereafter, the caudal ligature was tightened; a knot was made close to the artery on the excess length of the catheter, which subsequently was cut. The incision was closed over the catheter using a continuous pattern for the subcutaneous tissue and interrupted sutures for the skin (Vicryl 3/0 Ethicon). The cardiac catheter remained in place for the entire duration of the study.

The bacterial inoculum was prepared as described previously (28), and inoculation performed on sedated animals. The bacterial suspension (1 ml/kg) was injected into an ear vein over 1 min, followed by flushing of the vein with sterile isotonic saline. At 30 min after the injection of sedative, the pig received an intramuscular injection (0.04 ml/kg) of atipamezole hydrochloride (Antisedan; 5 mg/ml; Orion Pharma, Espoo, Finland) and was maintained under surveillance until fully awake. Heart rate, electrocardiogram and oxygen saturation were measured continuously during inoculation, and pigs were monitored closely for clinical signs of disease throughout the study. If any signs of discomfort were seen, the pig was given an intramuscular injection of buprenorphine (Temgesic 0.3 mg/ml; Shering-Plough, Heist up den Berg, Belgium) at 6 h intervals.

At euthanasia, the pigs were sedated with the sedative mixture, anesthetized with propofol, and subsequently exsanguinated by severing the large axillary and femoral blood vessels.

Echocardiography

All pigs were examined by the same experienced ultrasonographer using a Vivid 7 ultrasonographic system with a 5S multifrequent phased array transducer (2.2-5 MHz, octave imaging) on the day of surgery, and continuous electrocardiographic monitoring in association with the withdrawal of blood samples. Alcohol and an ultrasonic gel were used to ensure a good contact between the probe and the skin. The pigs were sedated, anesthetized with propofol, and examined from below while in lateral recumbency over a cut-out hole in the examination table. Right parasternal two-dimensional long-axis and short-axis views were used to image multiple tomographic planes. Spectral Doppler and color-flow imaging were also used in all pigs. The structures in proximity of the catheter in the heart were meticulously scrutinized for irregular-shaped echogenic masses adherent to the heart valves or mural endothelial surface. New valvular regurgitation

Table II: Summary of the experimental results.

Group	Pig number	NBTE	Staphylococcal endocarditis	Renal pathology	Blood cultures (cfu/ml)
A	A-1	+	-	-	0
	A-2	+	-	-	0
B	B-1	+	-	-	0
	B-2	+	-	-	0
C	C-1	+	-	-	0
	C-2	+	-	-	0
D	D-1*	-	-	+	0
	D-2	+	-	+	0
E	E-1	+	-	+	0
	E-2	+	-	-	0
F	F-1	+	-	-	0
	F-2	+	-	+	0
G	G-1	-	+	+	>300 ^a
	G-2	-	+	+	85 ^a , 14 ^b

*This pig presented with a non-thrombotic endothelial reaction and infiltrating inflammatory cells in the aortic valve.
^a, 24 h PI; ^b, 48 h PI.

was assessed by color-flow imaging of both valves on the left side of the heart. All echocardiographic examinations were digitally stored for later analysis by use of specialized software (EchoPac for PC, 7.0; GE Healthcare)

Bacteriology: examination of blood and organs

Blood sampling for bacterial cultivation was performed before start of the study, on the day of surgery, and at one, two, and four days PI. For the non-inoculated animals, blood sampling was performed before the study, on the day of surgery, and on the day of euthanasia. Blood was sampled from the left jugular vein in heparin tubes (NH170 IU; BD Vacutainer, Plymouth, UK) and kept at 5°C for a maximum of 4 h until processing.

Three 1-ml aliquots of blood were mixed with melted blood agar base (Oxoid Blood Agar Base, Oxoid, Basingstoke, UK) in Petri dishes. After solidification, the cultures were incubated at 37°C for two days. The number of *S. aureus* colonies was counted, isolated, and phenotypically characterized using Api Staph (Biomérieux, Inc., Marcy l'étoile, France).

Post mortem, a quantitative bacteriological examination was performed on lung tissue (margo dorsalis of the left diaphragmatic lobe). The surface of the organ was decontaminated by instantaneous

immersion in boiling water. From the interior of the organ a piece of tissue was weighed into a stomacher bag. A 10-fold volume of sterile isotonic saline was added to the bag and the contents were homogenized for 30 s using a stomacher. Thereafter, 10 µl, 50 µl, and 1 ml samples of the homogenate were mixed with melted blood agar in a Petri dish. The cultures were incubated at 37°C for two days, and the number of *S. aureus* colonies was counted.

Post-mortem examination

After euthanasia, the animals were subjected to necropsy and the organs sampled for histology, fixed in 10% neutral buffered formalin, processed through graded alcohols and xylene, and embedded in paraffin wax. Tissue sections were cut at 3-4 µm and stained with hematoxylin and eosin (H&E) (29). For *in situ* identification of *S. aureus*, immunostaining was performed as described previously (28).

Results

Clinical findings

During the period between catheter placement and bacterial challenge, none of the animals showed any clinical abnormalities. Furthermore, all non-inoculated animals (Groups A-C, Table I) remained clinically unaffected through the remainder of the study, as did

the pigs of groups E and F, and pig D-2. Pig D-1 developed a lameness of the left front leg after the second inoculation. In animals receiving a high dose of the porcine *S. aureus* strain (Group G), distinct clinical signs were seen on the day after inoculation. Fever, lameness and a reluctance to move was seen in pig G-1, and treatment with buprenorphine provided only a slight improvement. The pig was euthanized approximately 24 h PI, as the body temperature continued to rise. The other pig (G-2) had a similar, though slightly more protracted, clinical reaction and was euthanized at 48 h PI.

Echocardiography

A new valvular regurgitation of the aortic valve was seen in all pigs immediately after surgical placement of the catheter, and was also present in later echocardiographic examinations. When the endocardial thrombotic lesions reached approximately 2-3 mm in size, they could be detected echocardiographically as echogenic irregularly shaped thrombi close to the intracardiac catheter. An echocardiographic image of aortic vegetations in pig G-2 is shown in Figure 1.

Gross pathology and histopathology

All non-inoculated pigs developed aseptic thrombi, either valvular, mural, or both. The lesions were placed adjacent to the catheter and were millimeter-sized whitish elevations. Histologically, these lesions consisted primarily of an endothelial disruption with an overlying endocardial thrombus (Fig. 2). Endothelial hypertrophy of the endocardium in relation to the non-infected thrombi was also a common finding. The pigs of Groups D, E and F (except for pig D-1) presented the same endocardial lesions (NBTE) as the non-inoculated pigs. Pig D-1 presented endothelial hypertrophy and a subendothelial infiltration of inflammatory cells, mainly lymphocytes and macrophages in the aortic valve, and no fibrinous deposits. Furthermore, the animal had a myocardial infarction in the right ventricular wall, that was 2 × 2 cm in size.

The most distinct gross cardiac lesions were present in pigs of Group G (Table II). Pig G-1 had a whitish thrombus on a mitral valve cusp, with a size of approximately 1 cm, and a mural thrombus of approximately 0.5 cm (Fig. 3). There also was a thrombotic lesion on the aortic cusp. In pig G-2, there was a thrombus on the aortic leaflet (ca. 0.5 cm) and a thrombus of similar size immediately next to the aortic valve. Histologically, these lesions exhibited the classical organization present in IE (Figs. 4 and 5). On the endocardium, facing the cardiac lumen, was a pronounced layer of fibrin with numerous embedded

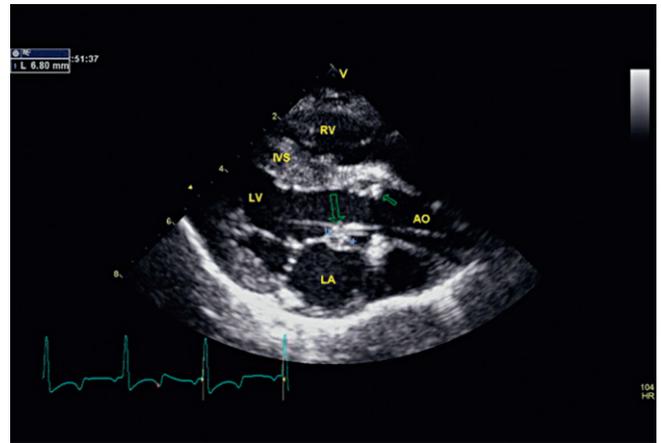


Figure 1: Right parasternal 5-CH long-axis view demonstrating a vegetation under the aortic valve (large arrow) and local thickening of the aortic valve (small arrow) in pig G-2 on day 2 PI. Note the catheter, which is in close contact with the 6.8 mm-diameter vegetation in the left ventricular outflow tract (large arrow). AO: Aorta; IVS: Interventricular septum; LA: Left atrium; LV: Left ventricle; RV: Right ventricle.

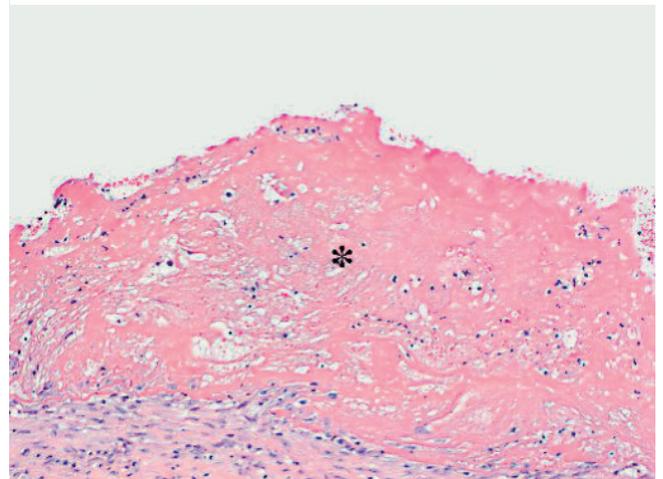


Figure 2: Non-bacterial thrombotic endocarditis in pig F-2 (*).

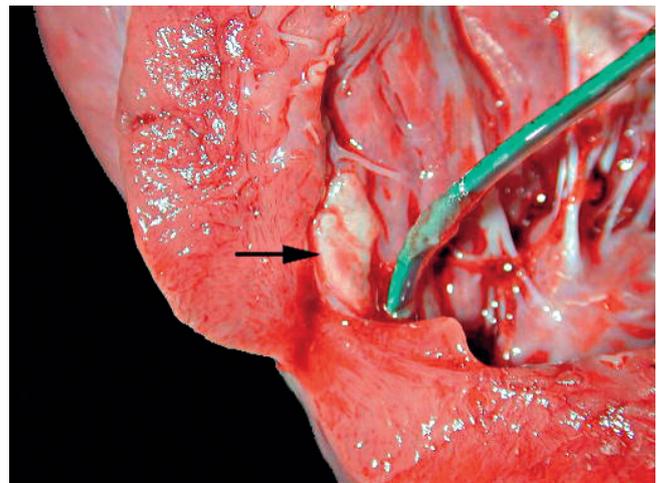


Figure 3: Left cardiac ventricle of pig G-1. Infected mural thrombus (arrow).

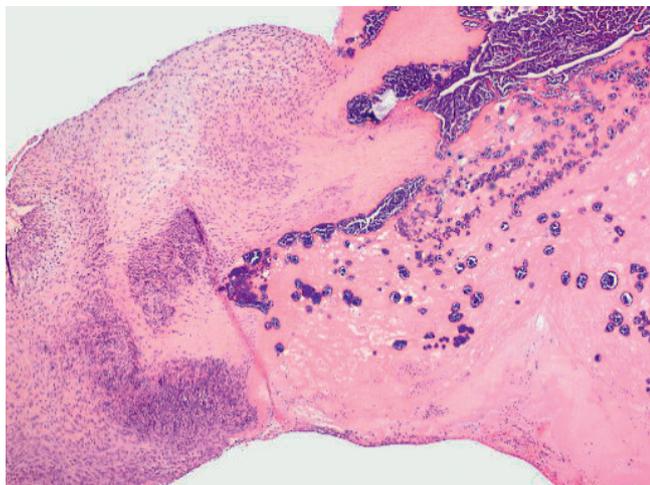


Figure 4: Cusp from the aortic valve of pig G-2. Staphylococcal endocarditis (H&E staining).

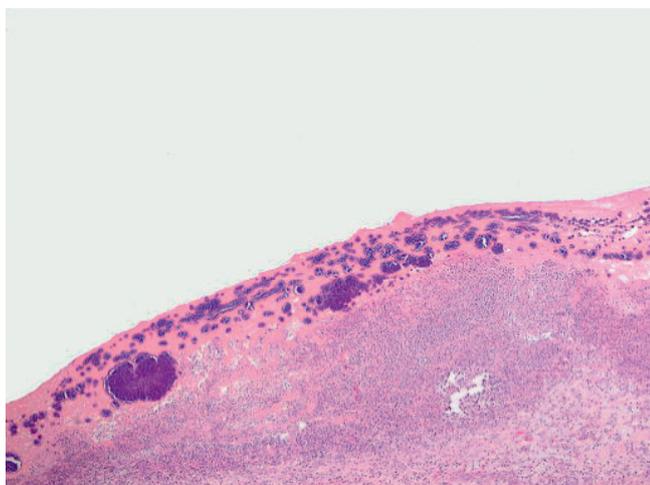


Figure 5: Staphylococcal endocarditis in pig G-1 (H&E staining).

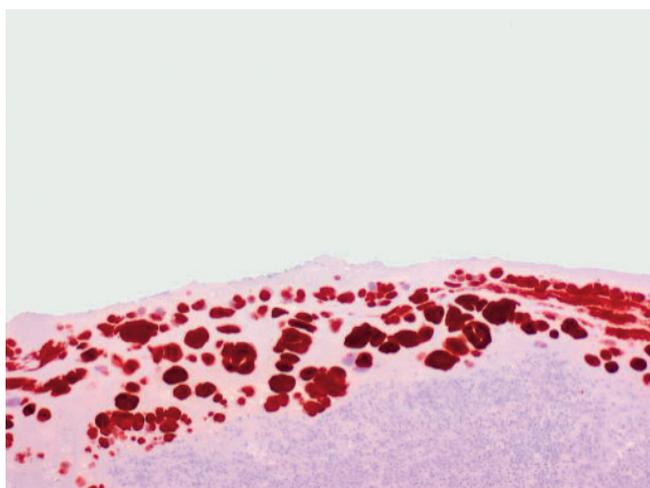


Figure 6: Staphylococcal endocarditis in pig G-1. Immunohistochemical staining for *S. aureus* reveals multiple positive colonies in the endocardial vegetation.

colonies with coccoid bacteria. Peripheral to this was an infiltration of inflammatory cells, mainly neutrophils, and a few macrophages. Pig G-2 also had multiple barely visible thrombi located murally and on mm. papillares, and disseminated myocardial microabscesses.

Immunohistochemical staining for *S. aureus* revealed the presence of bacteria embedded in the fibrinous material in the pigs of Group G only (Fig. 6).

Extracardial gross lesions were observed in the lungs and kidneys. One small abscess (1 mm in diameter) was seen in the left lung of pig D-1. Pig G-1 had disseminated abscesses (ca. 1 mm) in both lungs. All bacterially inoculated animals, except for pigs E-2 and F-1, had single or multiple renal infarcts, and pigs of group G also had small millimeter-sized disseminated renal abscesses. Pig F-2 exhibited a single abscess of similar size in the left kidney. None of the non-inoculated animals had any signs of embolization, or any other significant gross lesions.

Bacteriology

Prior to the bacterial challenge, all blood cultures were sterile. After inoculation, the blood cultures were positive from pigs in Group G (Table II). Pig G-1 had a bacterial blood count of >300 cfu *S. aureus* per ml blood on day 1 PI, when it was euthanized. In the case of pig G-2, the bacterial blood count was 85 cfu/ml on day 1 PI and 14 cfu/ml on day 2 PI.

Blood cultures were negative in all other animals at all times. *S. aureus* was also demonstrated in the lung tissues of pigs D-2, G-1, and G-2.

Discussion

In the present model, NBTE was catheter-induced in all but one of the animals. Furthermore, an inoculum of 10^7 cfu/kg of the porcine *S. aureus* strain S54F9 resulted in successful infection of non-bacterial thrombi with subsequent development of IE. This model of *S. aureus* endocarditis resembles the human disease in that the catheter causes damage to the endothelial layer of the endocardium, with subsequent development of NBTE. In the majority of human cases of IE, NBTE seems to be an essential nidus for the development of endocardial infection (30), where blood-borne bacteria from different sources then can attach to the thrombus and cause IE. This pathogenesis is relevant, as between 33% and 78% of healthcare-associated *S. aureus* bloodstream infections - that develop in >48 h following admission to a hospital or other healthcare facility, or within four weeks of an invasive procedure (31) - are attributed to intravascular devices which can cause NBTE formation and later IE (25). Furthermore, since the

cardiac catheters remained in place until euthanasia, this rendered both echocardiographic and pathologic information of vegetation placement in relation to the catheter. The tight correlation between intravascular devices and the development of IE, and the recognition of *S. aureus* as the leading cause of IE (4,5) could make this model - mimicking the human pathogenesis and etiology, and taking advantage of similarities between human and porcine physiology and anatomy - a valuable tool for the future study of IE in humans.

In the bacterially inoculated pigs, a dose and strain-dependent endocarditis response was found; that is, animals challenged with 10^7 cfu/kg of the S54F9 *S. aureus* strain developed clinical and pathological signs of IE. In contrast, pigs inoculated with 10^5 and later 10^6 cfu/kg showed no signs of IE. The size of the inoculum required to produce IE can vary depending on bacterial species and strains (32), which was also confirmed in the present model, as none of the animals challenged with NCTC8325-4 developed IE.

The required inoculum for inducing IE was higher in this porcine model compared to the rabbit model, in which a general susceptibility of 100% has been reported to be obtained with the use of inocula of at least 10^6 staphylococci, in animals having cardiac catheters in place for between two and seven days (32). One explanation could be differences in the pathogenicity of the different bacterial strains. Furthermore, the lung clearance of blood-borne organisms is quite host-dependent, and as the bacteria were inoculated intravenously the lungs were passed before reaching the left side of the heart. The porcine lung has a high capacity for retaining bacteria from the circulation, with a single-pass pulmonary clearance of 60-80 % over a wide range of infusion concentrations (33), whereas in rabbits the pulmonary bacterial clearance has been reported as minimal (34).

In the study of experimental porcine staphylococcal endocarditis described by Geissinger et al. (22), the bacterial inoculum was considerably higher than in the present study, ranging from 2×10^9 to 1.8×10^{11} staphylococci inoculated subcutaneously. Neither bacterial blood cultures nor echocardiographic examination was performed in that study, which means that two of the most valuable diagnostic tools for IE in humans were not evaluated in the model. The induction of experimental *S. aureus* endocarditis in pigs with intracardiac catheters has not been described previously.

The myocardial abscesses seen in pig G-2, and the renal abscesses in pigs of group G, were probably caused by an embolic event from the infected thrombi. Embolic complications are common findings in patients with IE (1).

In the pigs developing staphylococcal endocarditis, the clinical course was acute, with typical clinical signs, such as high-grade fever. This aggressive course was in good correlation with IE caused by *S. aureus* in humans, where the bacterium typically causes an IE that starts suddenly and progresses aggressively (35). Furthermore, the pigs with staphylococcal endocarditis both had positive blood cultures, which is an important diagnostic feature in human IE as an indicator of persistent bacteremia (36).

The demonstration of endocardial vegetations by echocardiography is an invaluable tool for the diagnosis and evaluation of IE in humans (36). In the present study, echocardiographic guidance was vital for surgical placement of the cardiac catheter, and was also used to diagnose both sterile and infected endocardial thrombi. Sterile thrombi were not distinguished from infected vegetations by echocardiography alone. However, together with clinical signs and isolation of viable *S. aureus* from blood samples, the diagnosis was clear.

In conclusion, non-bacterial thrombotic endocarditis and IE associated with *S. aureus* were successfully established in the present porcine models. The incidence of IE was dependent on the bacterial strain and inoculum size. Both, NBTE and IE revealed multiple similarities with human disease, and the porcine models should therefore be useful for future experimental studies.

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