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The domestic pig as a potential model for Borrelia skin infection

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ABSTRACT

The skin lesion erythema migrans is a characteristic early manifestation of Lyme borreliosis in humans. However, the pathomechanisms leading to development of this erythema are not fully understood. Models that mimic human skin would enhance research in this field. Human and porcine skin structures strongly resemble each other. Therefore, we attempted to induce erythema migrans lesions in experimental *Borrelia burgdorferi* sensu lato infection in the skin of domestic pigs. The formation of erythema migrans-like lesions was observed after intradermal injection of these spirochetes, with the lesions forming very clearly in 2/6 animals when a strain of *B. garinii* was used. However, no molecular or clinical proof of systemic infection of the pigs with *B. afzelii*, *B. burgdorferi* sensu stricto, or *B. garinii* could be achieved.

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1. Introduction

Lyme borreliosis (LB) caused by *Borrelia burgdorferi* sensu lato (Bbsl) is the most frequent tick-borne disease in the northern hemisphere (Stanek et al., 2012). In central Europe these spirochetes are transmitted by the hard tick *Ixodes ricinus* (Gern and Humair, 2002). The Bbsl species complex comprises at least 19 genospecies, including *B. burgdorferi* sensu stricto (Bbss), *B. afzelii*, *B. garinii*, *B. spielmanii*, and *B. bavariensis*, which are frequently found in human cases of LB (Mannelli et al., 2011; Stanek and Reiter, 2011). Interestingly, whereas in the United States LB is almost exclusively caused by Bbss, in Europe infections result from different species of Bbsl, with LB being mainly caused by *B. afzelii* and *B. garinii*, and to a lesser extent by Bbss (Stanek et al., 2012). One of the characteristic disease manifestations in humans is erythema migrans (EM), an expanding skin lesion usually forming within days after a bite by an infected tick (Hofhuis et al., 2013; Stanek et al., 2012; Strle and Stanek, 2009). After transmission of the spirochetes into the skin, the im-mune response of the host is triggered (Vig and Wolgemuth, 2014). Initially, the spirochetes are recognized by residing dendritic cells and sentinel macrophages via their pattern recognition receptors, leading to the production of pro-inflammatory chemokines and cytokines (Radolf et al., 2012). As a result, macrophages, plasmacytoid and monocytoid dendritic cells, neutrophils, and T-cells are recruited to the infection site (Radolf et al., 2012). The infiltrates of EM lesions predominantly consist of lymphocytes, with T-cells heralding the formation of the lesion (Radolf et al., 2012; Salazar et al., 2003). The hyperemia in the locally inflamed skin can manifest as rash (Vig and Wolgemuth, 2014). However, there is relatively limited understanding of the dynamics of EM development, and the factors responsible for expansion of the lesion are not fully determined.

Certain inbred strains of mice are commonly used as model animals for different aspects of Lyme borreliosis (Radolf et al., 2012). However, inbred mice strains that develop EM do not exist (Radolf et al., 2012). Rabbits have been shown to develop an EM lesion upon injection with *B. burgdorferi* (Foley et al., 1995). Rhesus macaques have served as a model for Lyme neuroborreliosis and appear to develop EM but are very expensive experimental animals (Pachner et al., 1995; Philipp et al., 1993; Radolf et al., 2012; Ramesh et al., 2015). We considered pigs as having the potential to be suitable models, as porcine skin shares a number of striking similarities with human skin (Summerfield et al., 2014). Among these are the

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thickness of the epidermis and the epidermal-to-dermal thickness ratio (Meurens et al., 2012). Moreover, pigmentation, hair follicle content, dermal blood supply, and general histology are comparable (Summerfield et al., 2014). In addition, dermal collagen and the elastic content of pig skin also resemble human skin to a considerable extent (Swindle et al., 2012). For these reasons pigs commonly serve as models for wound healing and have been used in studies on skin burns, transdermal penetration, toxicology, allergy, infectious diseases, and many others (Summerfield et al., 2014).

To our knowledge pigs have not been used as a model for human LB. Although strains of Bbsl have been shown to be sensitive to serum derived from pigs (Kurtenbach et al., 1998), more recent findings indicate the possibility of pigs being at least exposed to or even infected with these spirochetes. Thus, in a serologic survey in the Czech Republic, 12.8% of the tested sera from wild boar (*Sus scrofa*) contained antibodies against Bbsl (Juricová and Hubálek, 2009). Furthermore, in Portugal, borrelial DNA was detected in the serum of 3 out of 90 (3.3%) wild boar tested using PCR (Faria et al., 2015). Phylogenetically, European wild boar and European breeds of domestic pigs are very closely related and share identical haplotypes (Larson et al., 2005). Therefore, we concluded that experimental infection of domestic pigs with Bbsl is plausible.

2. Methods and materials

2.1. Experimental animals

The project required 18 healthy Large White pigs weighing 20–30 kg. One or two days before the start of the experiment the animals were cleaned and shaved at the intended injection sites. Immediately before the experiment the pigs were carefully cleaned again and the intended injection sites were disinfected several times with 70% ethanol before intradermal injection of 100 μL bacterial suspension containing either a high dose ($\sim 1 \times 10^9$) or a low dose ($\sim 1 \times 10^6$) of spirochetes per injection site. The mean *Borrelia* load of adult ticks is ranging from 5×10^3 to 1.8×10^6 Bbsl cells (Wang et al., 2003; Wilhelmsson et al., 2013). We used high cell counts for needle inoculation since the infection supporting factors of tick-saliva were missing (Radolf et al., 2012).

The following parameters were monitored on a daily basis throughout the project: average behavior with special respect to any neurologic abnormalities, inner body temperature, and gait with focus on possible joint disorders or lameness. Bacterial injection sites were monitored dermatologically. In addition, electrocardiograms were recorded on days 0, 1, 3, and 10. After a 10-day observation period, general anesthesia was induced with an intravenous bolus of 10 mg/kg ketamine and 1.5 mg/kg azaperone; the pigs then received a lethal intracardiac injection of T61®. Following euthanasia, a gross pathologic examination was performed and samples of ears, brain, cerebrospinal fluid (CSF), lungs, heart, bladder, kidneys, liver, spleen, jejunum, ileum, and colon were taken for histologic and microbiologic analyses.

2.2. Bacterial strains and culture conditions

The Bbsl strains used in the study were all of proven infectivity for humans. *B. burgdorferi* 375/10 and *B. garinii* 1460/12 both were obtained from human CSF, whereas *B. afzelii* 1870/12 was isolated from human blood. The strains are listed in Table 1. Strains were grown at 34 °C in a modified BSK II medium as previously described (Reiter et al., 2015). Fresh medium (10 mL) was inoculated with a frozen glycerol culture and bacteria were grown to logarithmic phase. This culture was used to inoculate a 50 mL culture at 1:5 dilution. After the bacteria were again grown to logarithmic phase, the culture was used to start a 250 mL culture (again at 1:5 dilution) and strains were grown to late logarithmic phase. *In vitro* passages were kept as few as possible. On the day of the experiment the spirochete cell count was determined by dark-field microscopy using a Petroff-Hausser or Neubauer counting chamber. Thereafter, the respective volume of the *Borrelia* culture was centrifuged at $6000 \times g$ for 10 min and the resulting pellet was resuspended in 6 mL of unsupplemented M199 medium (Sigma-Aldrich, Vienna, Austria). The bacterial suspension was then transported to the animal facilities and again centrifuged at $6000 \times g$ for 10 min in the local laboratory. For injection, the pellet was resuspended in fresh unsupplemented M199 medium to result in 1×10^9 or 1×10^6 spirochetes per 100 μL injection volume.

Table 1
Overview over the animals and Bbsl strains used in the study. Eighteen male pigs were injected with three strains of Bbsl: *B. burgdorferi* 375/10 and *B. garinii* 1460/12 were originally isolated from human CSF; *B. afzelii* 1870/12 is an isolate from human blood. High and low infectious doses contained 1×10^9 and 1×10^6 bacteria/injection site, respectively. Experiments were performed in four different experimental setups. All Bbsl strains were obtained from the University of Ljubljana (courtesy of Dr. Eva Ruzic-Sabljic).

	Animal #	Bbsl strain received	Dose received
Setup 1	1	*B. burgdorferi* 375/10	high
	2	*B. burgdorferi* 375/10	low
	3	*B. garinii* 1460/12	high
	4	*B. garinii* 1460/12	low
Setup 2	5	*B. burgdorferi* 375/10	high
	6	*B. burgdorferi* 375/10	low
	7	*B. garinii* 1460/12	high
	8	*B. garinii* 1460/12	low
Setup 3	9	*B. burgdorferi* 375/10	high
	10	*B. burgdorferi* 375/10	low
	11	*B. garinii* 1460/12	high
	12	*B. garinii* 1460/12	low
Setup 4	13	*B. afzelii* 1870/12	high
	14	*B. afzelii* 1870/12	high
	15	*B. afzelii* 1870/12	high
	16	*B. afzelii* 1870/12	low
	17	*B. afzelii* 1870/12	low
	18	*B. afzelii* 1870/12	low

2.3. Application of the spirochetes to the animals

Triplicate pigs were challenged with three Bbsl strains at the two inoculation doses. Experiments were conducted in four individual experimental setups, each on a different date, with three sets of 4 pigs each, and one set of 6 pigs. An overview of the animals and Bbsl strains is given in Table 1. Within a time frame of 20 min, an individual pig was injected intradermally with the respective dose at four sites making up a total inoculum of 4×10^6 or 4×10^9, respectively. The four inoculation sites were distributed on one mid-thoracic area, and three sites approximately 5 cm apart on the contralateral mid-thoracic area. Pure culture medium was inoculated as a control.

2.4. Sampling of blood, skin and other sites

Blood samples from the jugular vein were collected in EDTA-coated tubes on day 0 immediately before injection of the spirochetes and on days 1, 3, and 10 after inoculation. Plasma was obtained by centrifugation of the tubes at $600 \times g$ for 10 min. For blood culture, 1 mL plasma, including the buffy coat, was inoculated into 5 mL of bacterial growth medium containing rifampicin (50 μg/μL; SERVA, Heidelberg, Germany) and fosfomycin (100 μg/μL; Sandoz, Vienna, Austria). An ADVIA® 120 with ADVIA® 120 multi-species software version 3 3.1.8.0-MS (Siemens

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