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Experimental infection of chicken embryos with recently described *Brucella microti:* Pathogenicity and pathological findings

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ABSTRACT

Brucellae are facultative intracellular pathogens causing disease in a wide range of domestic and wild animals as well as in humans. *Brucella (B.) microti* is a recently recognized species and was isolated from common voles (*Microtus arvalis*), red foxes and soil in Austria and the Czech Republic. Its pathogenicity for livestock and its zoonotic potential has not been confirmed yet. In the present study 25 SPF chicken embryos were inoculated at day 11 of age with 1.6×10^3 and 1.6×10^5 *B. microti* by yolk sac and allantoic sac routes. Re-isolation of *B. microti* indicated rapid multiplication of bacteria (up to 1.7×10^{12} CFU). *B. microti* provoked marked gross lesions, i.e. hemorrhages and necroses. All inoculated embryos were dead (100% mortality) in between 2nd and 4th day post inoculation. The predominant histopathological lesion was necroses in liver, kidneys, lungs, spleen, gastrointestinal tract, spinal meninges, yolk sac and chorioallantoic membrane. Immunohistochemical examination showed the presence of *Brucella* antigen in nearly all of these organs, with infection being mainly restricted to non-epithelial cells or tissues. This study provides the first results on the multiplication and pathogenicity of the mouse pathogenic *B. microti* in chicken embryos. These data suggest that, even though chicken are not mammals, they could provide a useful tool for understanding the pathogenesis of *B. microti* associated disease.

1. Introduction

Brucellae are facultative intracellular, Gram-negative, non-motile, non-spore forming, aerobic pathogens and are causing disease in humans and a wide range of domestic and wild animals [1]. Based on host specificity and antigenic characters, the genus *Brucella* (B.) is currently divided into 11 nomo-species. The six 'classical' species are *B. melitensis*; *B. abortus*; *B. suis*; *B. canis*; *B. ovis* and *B. neotomae* [2]. Recently, four new species were described. Two of them are of marine origin: *B. pinnipedialis* and *B. ceti* [3], and the other two species are *B. microti* isolated from the common vole *Microtus arvalis* [4], and *B. inopinata* isolated from a breast implant wound of a female patient [5]. Finally, *B. papionis* was described from an isolate from baboons (*Papio* spp.) [6]. The virulence mechanisms of *Brucella* spp. in a given host species are not completely known [7]. The most important aspect of virulence is the ability of the bacterium to proliferate and survive inside of phagocytic and non-phagocytic cells avoiding an immune response of the host [8]. *Brucellae* that invade livestock are restricted to specific hosts to some extent. For example, small ruminants, bovines, pigs and sheep are classical and preferred hosts for *B. melitensis*, *B. abortus*, *B. suis* and *B. ovis*, respectively [7]. Nevertheless, the mechanisms that permit host cells to be invaded by *Brucella* spp. are not completely understood and the specific host receptors towards certain *Brucella* spp. have not been investigated yet.

B. microti was originally isolated from systemically diseased common voles (*Microtus arvalis*) captured in the Czech Republic in 2000 and had been isolated also from red foxes and soil [4,9,10]. *B. microti* is characterized by marked metabolic capabilities and fast growth on standard media [4]. Isolation of *B. microti* directly from soil reflects its ability to persist for a long time in an environment outside mammalian hosts. Diversity of reservoir species of *B. microti* may also play an important role in the epizootic spread of

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Table 1
Sampling protocol and mortalities of chicken embryos inoculated with *B. microti* at day 11 of age by different routes and dosages.

Groups	Number of embryos	Age of inoculation	Dosage CFU/egg	Route of inoculation	Mortalities/days post-inoculation[a]					Total mortality
					1st day	2nd day	3rd day	4th day	5th day	
Group 1	5	11 days	1.6×10^3	Yolk sac	0	2	2	1	0	5/5
Group 2	5	11 days	1.6×10^3	Allantoic sac	0	0	3	2	0	5/5
Group 3	5	11 days	1.6×10^5	Yolk sac	0	2	3	0	0	5/5
Group 4	5	11 days	1.6×10^5	Allantoic sac	0	0	5	0	0	5/5
Group 5	5	Negative control			0	0	0	0	0	0/5

[a] Number of dead embryos post inoculation.

this species [11]. The pathogenicity of *B. microti* for humans and livestock has not been investigated and remains unknown [12]. Although Brucellae are not considered as pathogens for poultry, this study was designed to investigate the effect of experimental infection of chicken embryos (CE) by the newly recognized *B. microti*. This could be helpful to study the pathogenesis of *B. microti* associated changes in chicken embryos, identify the preferential tissues and cells invaded by *B. microti* and could be supportive to establish an in ovo infection model for *Brucellae*.

2. Materials and methods

2.1. Bacterial strain and colony forming unit (CFU) determination

The strain used in the present work was *B. microti* CCM 4915 (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Germany). Bacteria were grown on tryptic soy media for 48 h and then washed with phosphate buffered saline (PBS). CFU was determined by plating serial dilutions onto tryptic soy agar plates.

2.2. Chicken embryo inoculation

Specific pathogen free eggs used in this study were obtained from VALO SPF flocks, Bio Media, Osterholz-Scharmbeck, Germany. The eggs were delivered one day before the start of the experiment. All eggs were incubated in an egg incubator at 37 °C and a relative humidity of 50–60%. At day 11, all incubated eggs were candled and only living embryos were used for the study. A total of 25 eggs were used in this study and were divided into five groups (each with $n = 5$) according to the different doses and routes of infection. Freshly harvested *B. microti* suspended in PBS were used for inoculation (0.2 ml inoculum volume). By candling, the air chamber was detected and the outer shell area was disinfected with iodine. The air chambers of the eggs were perforated with a sterile needle distant from blood vessels. Lower doses of 1.6×10^3 CFU were inoculated into the yolk sac (group 1) or the allantoic sac (group 2). Higher doses of 1.6×10^5 CFU were applied to the yolk sac (group 3) or the allantoic sac (group 4). Group 5 was used as a negative control and inoculated with PBS (Table 1). The shell perforation was sealed with melted white wax. Afterwards, the eggs were returned back into the incubator. CEs were checked by candling three times a day post inoculation with an interval of 8 h to observe the viability of the embryos. Mortality rate was recorded and dead embryos were sampled just after death (Table 1). At the end of the experiments the control group was sacrificed by keeping at 4 °C till death (2 h).

2.3. Processing of samples for bacteriology

Amniotic fluid was collected from all freshly dead embryonated eggs under sterile conditions and subjected to bacterial isolation, counting and identification. Bacterial re-isolation and cell count were carried out on blood agar and tryptic soy agar plates at 37 °C for 48 h. Cell count was assessed by plating serial dilutions onto tryptic soy agar plates. Bacterial identification was determined by a modified genus-specific *Brucella* PCR based on BCSP31 as described elsewhere [13].

2.4. Histopathology

The trunks as well as the chorioallantoic membranes and the yolk sacs of 22 embryos (all infected embryos and two of the negative control group) were fixed in 4% neutral buffered formalin for at least 24 h. Afterwards, each trunk was cut perpendicular to the longitudinal axis to provide 4 transverse planes for the following histopathological investigations. The segments of the trunks as well as representative samples of each chorioallantoic membrane (CAM) and yolk sac were embedded in paraffin, sectioned at 3–4 μm and stained with hemalum and eosin (HE). Hemalum is a mixture of the oxidation product of hematoxylin and aluminum ions that is used to stain cell nuclei [14]. Moreover, the heads of 2 embryos were fixed as described above. Subsequently, they were cut approximately in the median plane and one half of each head was embedded in paraffin followed by sectioning alongside a sagittal plane and HE staining of the samples.

The histology slides were screened for pathological changes, especially in the heart, lung/bronchi, liver, spleen, kidneys, gastrointestinal tract, integument, spinal cord, brain, eyes, mucosa of oral and nasal cavity, CAM and the yolk sac wall. Microscopic examination was focused on signs of inflammation and cell death. The term cell death is used in the following to group cellular alterations including hypereosinophilic cytoplasm, indistinct cell borders and nuclear damages such as karyorrhexis, karyopyknosis or karyolysis. Cellular alterations visible in the HE stained slides were grouped semiquantitatively as 'few', 'moderate numbers of' and 'numerous' affected cells. Based on the HE stained slides, representative specimens were chosen for Taylor's stain to demonstrate the presence of Gram-negative bacteria [15].

Statistical evaluation of survival rate and severity of the lesions was performed with Kaplan–Meyer survival analysis and Mann–Whitney-test, respectively.

2.5. Immunohistochemistry

Immunohistochemistry was performed on all paraffin-embedded samples of 10 embryos, i.e. two randomly chosen embryos from each of the four infection groups as well as the two embryos serving as negative controls.

The peroxidase anti-peroxidase (PAP) method was applied [16]. In brief, sections of the paraffin-embedded samples alongside the transverse planes as described above were dewaxed, rehydrated and endogenous peroxidase was inactivated by treatment with 3% hydrogen peroxide in methanol for 30 min at room temperature. Epitope demasking was achieved using citrate buffer. The polyclonal rabbit anti-*Brucella* primary antibody (Bioss, Woburn, MA, USA) was diluted 1:4000 in tris-(hydroxymethyl)-aminomethane-buffered saline (TBS). Sections were incubated overnight at 4 °C and rinsed thoroughly with TBS. Sections were then treated for 30 min