

Evaluation of the possibility of *C. burnetii* transmission by the alimentary route in a guinea pig model

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Abstract

Introduction: Q fever (coxiellosis) is an infectious disease of animals and humans, caused by *C. burnetii* and widely distributed throughout the world. It is known that people and animals acquire the disease predominantly *via* inhalation of infectious aerosols. The possibility of transmission of the pathogen by the alimentary route is still a matter of debate and remains controversial. Therefore the aim of this study was to fill the gaps in knowledge of oral transmission of *C. burnetii* by conducting biological tests on the guinea pig model. **Material and Methods:** Guinea pigs, divided into five groups comprising a negative control and four experimental groups, received specified concentrations of *C. burnetii per os*. To determine the presence of specific antibodies, blood samples were tested using CFT. Also, internal organs collected during necropsy were screened by a real-time PCR targeting IS1111. Additionally, histopathological evaluation of the tissues was performed. **Results:** The presence of antibodies and pathogen DNA in caecum was confirmed in one guinea pig from experimental group IV. *C. burnetii* was also detected in testicular tissue collected from one animal of experimental group II. **Conclusions:** The presence of pathogen DNA in the testicular tissue indicates that infection spreads haematogenously. In the majority of experimental animals specific antibodies and genetic material of *C. burnetii* were not detected. This fact suggests that development of infection depends on many factors, such as animal immune status.

Keywords: guinea pig, *Coxiella burnetii*, alimentary route, infection.

Introduction

Q fever is a widespread zoonotic disease caused by the obligate intracellular microorganism *Coxiella burnetii*. This Gram-negative bacterium can infect a wide range of hosts, including humans, wild and domestic mammals, birds, reptiles, and arthropods (1, 13, 21). Clinical symptoms of Q fever in both humans and animals are various and nonspecific, moreover infections often remain asymptomatic. The environment becomes contaminated with the bacteria mainly due to its excretion by infected ruminants (*i.e.* cattle, sheep, and goats). The highest numbers of *C. burnetii* are shed in birth products during abortion or parturition, and it is also passed out of the body in milk, faeces, urine, and semen (9, 14). It is assumed that people and animals contract the disease predominantly by inhalation of infectious aerosols or direct contact with affected individuals or contaminated animal products. Some

research indicates that the consumption of unpasteurised milk may be a cause of infection (17, 19), and the presence of viable *C. burnetii* was demonstrated in raw bovine milk (7, 12). Nevertheless, debate continues on this mode of transmission and a direct link between development of Q fever and ingestion of contaminated raw milk needs to be confirmed.

Since the discovery of *C. burnetii* in the 1930s, many animal models have been used to investigate the pathogenesis of Q fever and evaluate the protection abilities of developed vaccines (4). It has been demonstrated that immunocompetent mice are less susceptible to *C. burnetii* infection than guinea pigs (16). The latter species is known as a very good animal model of acute Q fever in humans, with fever being the primary indicator of the disease (8). In this study we attempted to fill the gaps in knowledge of oral transmission of the pathogen and utilised a guinea pig model to assess the morbidity and mortality after *per os* administration of

phase I *C. burnetii* Nine Mile strain. Taking into consideration the increasing consumption of raw milk and artisanal dairy products, this study is significant for the protection of the public's health.

Material and Methods

Strain cultivation and the preparation of infective inoculum. The African green monkey kidney (VERO) cells were cultivated in minimum essential medium (MEM) (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (PAN-Biotech, Germany) at 37°C in the presence of 5% CO₂. The phase I Nine Mile RSA493 strain, kindly provided by Wageningen University & Research (the Netherlands), was used to inoculate the cell line. The infected cells were passaged twice at seven-day intervals. The cytopathic effect and the number and size of phagolysosome-like vacuoles containing *C. burnetii* cells were microscopically assessed daily. After incubation, the culture media from all the bottles were collected and pooled. Then 12 mL of PBS was added to each bottle, the cells were scraped and obtained suspensions were also pooled. Collected medium and cell suspension were sonicated and centrifuged at 500 × g for 10 min in 15 mL Falcon tubes. The supernatant was collected into Eppendorf tubes and then centrifuged for one hour at 24,400 × g. Next, the supernatant was rejected and the remaining pellet was resuspended in 400 µL PBS per Eppendorf tube (sludge from the culture medium) or in 800 µL PBS per Eppendorf tube (cell suspension). This solution was subsequently filtered using sterile filters with pore sizes of 1.2 and 0.45 µm. To determine *C. burnetii* concentration after purification, 10-fold serial dilutions of the filtrate were prepared in sterile PBS (to 10⁻⁶) and stained with a LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Additionally, a real-time PCR targeting *com1* was performed as described by Lockhart *et al.* (11). Then suitable dilutions of bacteria were prepared, aliquoted, and frozen at -80°C. Before starting the experiment, the dilutions were defrosted and the concentration of viable bacteria was checked with the LIVE/DEAD® BacLight Bacterial Viability Kit. The cultivation of the *C. burnetii* strain was performed in BSL3 (Biosafety Level 3) laboratory conditions.

Guinea pigs. Fifteen 6–7-week-old in-house bred (NVRI, Poland) guinea pigs (*Cavia porcellus*) weighing 400–450 g were used in the experiment. Animals were housed in individual cages, each group in a separate room, in a BSL 3 facility with a 12 h–12 h light–dark cycle. The number of air exchanges was 12 per hour and temperature and humidity were maintained between 18–25°C and 40–60%, respectively. The animals were given water and commercial LSK guinea-pig feed (Agroprod, Poland) *ad libitum*. Vitamin C was administered at 10 mg/kg b.w. orally every day to prevent its deficiency. To reduce stress-related abnormalities, guinea pigs were acclimated to the facility for two weeks

prior to the experiment. They were randomly divided into five groups: a negative control group (n = 3) and four experimental groups (each n = 3). Prior to the start of the experiment, blood and faecal samples were collected from all animals for serological and molecular analyses to exclude the possibility of *C. burnetii* infection. The guinea pigs' health was assessed daily and rectal temperature and behaviour were recorded.

Infection. The experiment lasted 12 weeks. Guinea pigs in the control group received 1 mL of sterile PBS solution applied by syringe directly into the mouth every 48 hours. Animals from experimental group I received 1 mL of inoculum containing 10² of *C. burnetii* cells/mL once by the same route. Doses of 1 mL of 10² *C. burnetii* cells were administered *per os* every 48 hours to guinea pigs in experimental group II. Animals belonging to group III received 1 mL of bacterial suspension containing 10⁶ *C. burnetii* orally once. Individuals in group IV were given 1 mL of the inoculum containing 10⁶ bacterial cells every 48 hours. General physical examination of guinea pigs from all groups was carried out daily, and blood from all animals was collected to determine the titres of specific antibodies on days 5 and 7 and then weekly during the experiment. After 12 weeks the guinea pigs were euthanised by intravenous injection of pentobarbital sodium at a dose of 100 mg/kg b.w. Then the heart, lungs, liver, spleen, testes, stomach, caecum, and small intestine of all animals were collected during the autopsy.

Histopathology. Tissues collected at post-mortem examination were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5 µm slices, and routinely stained with haematoxylin-eosin (HE). Microscopic examination was performed using an Axiolab microscope (Zeiss, Germany).

Serology. Blood samples were stored at room temperature for 30–45 min to allow clotting and then centrifuged at 1,000 × g for 10 min to obtain serum. Then, serum specimens were tested for the presence of antibodies to *C. burnetii* using a complement fixation test (CFT). Detection of phase-specific IgM and IgG was performed utilising reagents supplied by Virion/Serion (Germany), and their instructions were followed in measuring antibody levels. The inactivation temperature of the sera was 56 ± 1°C, the initial dilution of the sample was 1:5, and the final dilution was 1:80. Serum was considered positive when at least partial inhibition of haemolysis (+) was observed in the dilution 1:10.

Real-time PCR. Extraction of DNA from faeces and tissue specimens collected at post-mortem examination was performed using a QIAamp DNA Stool Mini Kit and DNeasy Blood & Tissue Kit (Qiagen, Germany). Extraction procedures were performed as the manufacturer directed and DNA aliquots were stored at -20°C until use.

All DNA extracts were screened for the presence of *C. burnetii* IS1111 by real-time PCR according to Capuano *et al.* (5), including a commercial internal amplification control – TaqMan Exogenous Internal Positive Control (Thermo Fisher Scientific, USA) – to

Discussion

An inspiration for the study was limited and contradictory data about the possibility of *C. burnetii* transmission to humans by the alimentary route. Taking into account the large epidemic with more than 4,000 human cases of Q fever in the Netherlands from 2007 to 2010, the risk for public health is significant. Babudieri (2) refers to unpublished research indicating unsuccessful attempts at infection of 75 calves by giving them contaminated milk. The opposite result was obtained by Bell *et al.* (3), who infected two calves in the same way. The effectiveness of this oral inoculation was confirmed by the isolation of the pathogen from the calves' blood using guinea pigs. Durand (6) determined that in the case of mice, a 10,000 times higher *C. burnetii* dose is needed for alimentary than for intraperitoneal infection, whereas Kruszevska and Tylewska-Wierzbanska (10) effectively inoculated mice with the same dose of bacteria, independently of the route of infection. Schaal (15) confirmed the presence of specific immunoglobulins in 8 out of 25 guinea pigs which received milk containing *C. burnetii per os* twice a day. Interestingly, seroconversion was not observed in individuals of which the mucous membrane of the stomach was irritated mechanically or chemically. In five out of eight seropositive animals, the presence of a viable pathogen in the internal organs (testes and spleen) was confirmed by inoculation of laboratory animals. Moreover, clinical symptoms of Q fever were observed in some experimental groups, but their type and severity were not described. Interestingly, they were recorded in a group of animals which did not produce specific antibodies. It was also not mentioned whether attempts were made to isolate the pathogen using tissues derived from seronegative individuals.

In this study animals from experimental groups did not exhibit any clinical symptoms that could indicate *C. burnetii* infection; however, the presence of pathogen DNA in the testes of one animal was confirmed. Moreover, another guinea pig had high levels of specific antibodies and the bacteria were also detected in the caecum of this animal. Similarly to Schaal's experiment (15), pathogen genetic material was found in testicular tissue, which indicates that the gastrointestinal mucosa barrier was overcome and *C. burnetii* spread haematogenously. No specific antibodies were detected in the blood of this individual. Perhaps in guinea pigs, as in humans, the appearance of specific immunoglobulins is preceded by the presence of bacteria in the blood, which would explain the presence of *C. burnetii* in the testicular tissue. It seems interesting that the animals did not develop clinical symptoms of the disease and seroconversion was noted only in one guinea pig. *C. burnetii* as an intracellular pathogen may avoid the immune response, and the body's response to infection with this microorganism may depend on the immune status (18, 20). The presence of *C. burnetii* in the testicular tissue may suggest that the pathogen can infect the organism *via* the alimentary route, infiltrate the cells

and stay in them until there are favourable conditions for replication (*i.e.* immunosuppression). Research in this area should be continued including work using immunosuppressed animals as an experimental group. In order to evaluate the possibility of development of chronic disease, the experimental duration time might be prolonged. Moreover, genetically diverse *C. burnetii* strains might be used to investigate their pathogenic potential *via* the alimentary route.

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Animal Rights Statement: The experiments on animals were conducted in accordance with the local Ethical Committee laws and regulations as regards care and use of experimental animals.

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