Seroprevalence of *Coxiella burnetii* among domestic ruminants and horses in Poland

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Summary. *Coxiella burnetii* is the causative agent of Q fever. The cases of the disease are recorded in various species, including domestic animals. The aim of this investigation was to estimate the seroprevalence of *C. burnetii* in ruminants including cattle, sheep, goats, and horses. Totally, 2082 serum samples from 936 goats, 933 cattle, 89 sheep, and 124 horses, including various horse breeds, were tested by ELISA or complement fixation test. The examination revealed that Polish horses are seronegative while in the populations of cattle and small ruminants, seropositive animals are presented. The percentage of seropositive cattle, goats and sheep was 4.18, 6.30, and 13.48, respectively.

Keywords: Coxiella burnetii; domestic animals; horses; seroprevalence

Introduction

*Coxiella burnetii* is the causative agent of Q fever. Over the last few years, the number of Q fever cases have been increased throughout the world. The pathogen has been detected in various animal species while cattle and small ruminants i.e. sheep and goats are considered to be the primary hosts. However, high levels of seroprevalence and shedding were recorded in domestic animals in Europe (Astobiza et al., 2012; Muskens et al., 2011; Ryan et al., 2011), data from many countries are still underestimated or incomplete. Since 1956, Q fever in ruminants was regularly reported also in Poland, but the role of horses as a reservoir of *C. burnetii* have not yet been investigated. Transmission of *C. burnetii* to humans is possible through the inhalation contaminated aerosols or direct contact with animal excrements or with infected animals. Also, the possibility of infection by consumption of raw milk cannot be excluded (Signs et al., 2011). Due to the outbreak of Q fever in Netherlands between 2007 and 2010 which widespread this zoonotic agent into the environment and resulted in more than 4,000 human cases, the surveill-
Table 1. Serological examination of cattle and small ruminants herds by ELISA

<table>
<thead>
<tr>
<th>Province</th>
<th>Cattle</th>
<th>Small ruminants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of tested samples</td>
<td>No. of positive samples/</td>
</tr>
<tr>
<td>Dolnośląskie</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Kujawsko-Pomorskie</td>
<td>87</td>
<td>3/3.45</td>
</tr>
<tr>
<td>Lubelskie</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Lubuskie</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Łódzkie</td>
<td>75</td>
<td>3</td>
</tr>
<tr>
<td>Małopolskie</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mazowieckie</td>
<td>87</td>
<td>1/1.15</td>
</tr>
<tr>
<td>Opolskie</td>
<td>87</td>
<td>13/14.94</td>
</tr>
<tr>
<td>Podkarpackie</td>
<td>93</td>
<td>1/1.08</td>
</tr>
<tr>
<td>Podlaskie</td>
<td>20</td>
<td>1/5</td>
</tr>
<tr>
<td>Pomorskie</td>
<td>51</td>
<td>1/1.96</td>
</tr>
<tr>
<td>Śląskie</td>
<td>40</td>
<td>5/12.5</td>
</tr>
<tr>
<td>Świętokrzyskie</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Warmińsko-Mazurskie</td>
<td>92</td>
<td>10/10.87</td>
</tr>
<tr>
<td>Wielkopolskie</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>Zachodnio-Pomorskie</td>
<td>101</td>
<td>1/0.99</td>
</tr>
<tr>
<td>total</td>
<td>933</td>
<td>39/4.18</td>
</tr>
</tbody>
</table>

NA = not available.

from randomly selected herds excluding vaccinated animals. The bovine samples were investigated during the Multiannual Research Programme, while samples from horses and small ruminants were tested within the networking project titled “Q fever biomarker discovery using innovative immunoproteomic and metabolomic approaches” supported by Visegrad Fund. Detection of antibodies against _C. burnetii_ in cattle, and small ruminants were performed using commercially available indirect ELISAs (IDEXX) test according to the manufacturers’ instructions while horse sera were tested by CFT (Virion/Serion).

Results

All sera collected from horses were negative for antibodies to phase I and II antigens in complement fixation test (CFT). The presence of specific immunoglobulins against _C. burnetii_ was detected by ELISA in 39 (4.18%) bovine samples from 20 herds. The highest percentage of seropositive herds, more than 15.0%, was noted in Kujawsko-Pomorskie, Pomorskie and Warmińsko-Mazurskie provinces. The lowest proportion of positive bovine herds was recorded in Podkarpackie province (1.08%). The detailed data from individual regions are shown in Table 1. Among 936 tested goat sera, only 59 (6.3%) were identified as positive, and the number of seropositive herds was calculated as four (12.5%). The percentage of the seropositive sheep was 13.48% (12 of 89 tested animals). The seroprevalence rate in sheep herds was 66.66% (8 of 12 tested herds). Moreover, five of tested sheep samples from four flocks and 17 goat sera from two herds were suspect in ELISA test. The seroprevalence in small ruminants herds in the provinces are shown in Table 1.

Discussion

The first case of Q fever in Poland was recognized in 1956 in the flock of sheep, and the biggest outbreak in man took place in 1982 in the east of Poland. It affected more than 1000 humans who acquired the disease from infected dairy cattle herd (Cygan et al., 1986). During the last decades, only a few cases of this infection in humans and animals were reported in Poland, and thus the data are insufficient. Cattle are the most important livestock in the country with the population of 5.938 million in 2016. Therefore, it is not surprising that cattle were the primary source of the Q fever
outbreaks in Poland, whereas in general, the small ruminants are considered to be the main source of infection for humans (Chmielewski and Tylewicz, 2013). The seroprevalence among cattle herds in this study was significantly lower than reported in the previous survey (Jodełko et al., 2015) when samples were tested by CFT (Siemens Healthcare, Germany). The cause of decrease of seroprevalence has not yet been determined, but we speculate that it may be related to differences in diagnostic method. Several papers reported that some ELISA tests might vanish positive results in the case when the serum contains low titers of antibodies specific for phase II C. burnetii antigens (Emery et al., 2012; Szymańska-Czerwińska et al., 2016; Kittelberger et al., 2009). The second reason might be associated with effective control of Q fever i.e. by vaccination which was utilized from 2013 in Poland. Out of tested small ruminant, 27.27%, (12 of 44 tested) of herds were seropositive. It is impossible to compare this results with data from previous decades and years because there are only a few reports limited to the provinces (Cisak et al., 2003; Kneblewski et al., 2017).

The population of small ruminants in Poland is not as huge as cattle but ecology farms offering raw milk not only from the cattle but also from goats or sheep. In recent years the organic food market has been developing very fast. We have observed an increase in the production of food based on ecological raw materials e.g. milk. Therefore, the monitoring surveys of C. burnetii are justified. The significant role of ruminants as a reservoir and shedders of C. burnetii is well known (Mertens et al., 2017). In contrast, knowledge of infections in horses is limited, and usually, these animals are considered to be a potential source of this bacteria. Marenzoni et al. (2013) based on published data, calculated the pooled mean seroprevalence in this species to 15.8%. However, we did not confirm this assumption. According to our data, none of the tested equine blood samples were positive in CFT. Nevertheless, the presence of the C. burnetii without seroconversion cannot be excluded. Thus, this research needs further surveys focussing on detection of shedders among horses and ruminants that can be performed by any complementary method as PCR. This research is currently ongoing, and it will be published soon.

References


Two mice models for transferability of zoonotic bacteria via tick vector

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Summary. – Spotted fever and typhus-related diseases caused by rickettsiae, Lyme borreliosis induced by spirochetes from Borrelia burgdorferi sensu lato complex, and Q fever evoked by Coxiella burnetii, are important zoonoses occurring worldwide. In order to study the pathogenesis of these infections, the efficacy of vaccines from the perspective of protection against the pathogens, pathogen – pathogen interactions during co-infections or pathogen-vector-host interrelationship, a suitable animal model should be established. In this study, we evaluated two mouse models – the C3H/N and Balb/c strains for susceptibility to infection and ability to transmit the pathogens via tick vector and to reveal the potential interactions between various bacterial tick-borne agents. Our results indicated that the C3H/N and Balb/c mice are well-accepted models of Borrelia afzelii infection. However, they are not suitable for interaction studies with Rickettsia helvetica since the animals did not acquire rickettsiemia and do not transmit Rickettsia sp. to feeding ticks.

Keywords: animal model; pathogen transferability; tick vector; Ixodes ricinus; Coxiella burnetii; Rickettsia helvetica; Borrelia afzelii

Introduction

Tick-borne diseases that afflict animals and humans are caused by infectious agents transmitted by tick bites. Lyme borreliosis and rickettsioses belong to the most common tick-transmitted infections (Kurtenbach et al., 2006; Parola et al., 2013). The sheep tick, Ixodes ricinus is a geographically widespread species and the most common vector for the bacterium Rickettsia helvetica and spirochetes of the Borrelia burgdorferi sensu lato (s.l.) complex including Borrelia afzelii (Rizzoli et al., 2014). In this tick also Coxiella burnetii was detected (Rehn and Radvan, 1957; Špitalská and Kocianová, 2003; Hildebrandt et al., 2011). The infection may occur during a blood meal on infected animals, and the infection is then transmitted to other mammals during the next blood meal (Arricau-Bouvery et al., 2006; Široký et al., 2010). Therefore, the ticks are considered as important reservoirs and potential vectors of rickettsiae and C. burnetii, which are transstadially and transovarially transmitted in some tick species (Daiter, 1977; Klyachko et al., 2007; Široký et al., 2010).

Ticks can even harbour more than one disease-causing agent at the same time. During life cycle, I. ricinus feeds on three different hosts. Therefore the probability of infection with different pathogens is very high (Gray, 1984). Thus, inside the tick, pathogens might interact and affect each other during the natural vector-pathogen-host cycle (Václav et al., 2011). To study the transferability of the pathogens within the tick vector-animals-cycle and pathogen – pathogen interactions during co-infections, a suitable animal model must be established. Recent studies have reported that these infections can be established in mice, depending on the genetic background of mice, the individual tick-borne species and the route of inoculation (Bechah et al., 2008; Tonetti et al., 2015). Inbred mice were used to define haplotype susceptibility and to study the pathogenesis of arthritis or carditis evoked by borrelial infection. Also, the C3H mice have been employed to correlate genetic markers and development of chronic or late borrelial infection (Herrmann, 1995).

In order to shed light on the role of arthropod vectors within the pathogen circulation in natural foci, we tested...
two mouse models for *Rickettsia helvetica* and *Borrelia afzelii* infection. The specific goal was to determine the suitable animal model and figure out the transmission efficiency and potential of the interaction between bacterial pathogens during coinfection. The experiment was performed under laboratory conditions with C3H/N and Balb/c mice as reservoir hosts and *I. ricinus* ticks as a vector.

**Materials and Methods**

*The model host organism.* Thirty-five 8-week-old pathogen-free female C3H/N and Balb/c mice were used and housed individually according to EU guidelines. All animal protocols were approved by the Ethics Committee of the Institute of Veterinary, Biomedical Research Center SAS and the State Veterinary and Food Institute of the Slovak Republic (Permit No. 292/16-221b).

*Ticks.* Uninfected laboratory reared *I. ricinus* larvae originated from the laboratory colony of the Institute of Zoology SAS, Bratislava, Slovak Republic and Institute of Parasitology, Biology Centre CAS, České Budejovice, Czech Republic were employed.

*Bacterial pathogen.* The CB-43 strain (Štěpánová-Tresová et al., 1999) of *Borrelia afzelii* grown in BSK-H media and *Rickettsia helvetica* (Sekeyová et al., 2012) propagated on Vero cells were studied.

*Experimental design.* Two mouse strains, C3H/N and Balb/c, were used. Balb/c mice were separated into 4 groups with 5 mice each. The 1st and 2nd groups were inoculated with *B. afzelii* strain and *R. helvetica*, respectively. The 3rd group was inoculated with saline as negative control. The 2nd experiment included 20 C3H/N mice which were separated into four groups with 5 mice each (1st group – mice inoculated with *B. afzelii* strain, 2nd group – mice inoculated with *R. helvetica*, 3rd group – mice inoculated with *B. afzelii* strain and *R. helvetica*, 4th group – mice inoculated with saline as negative control group). Mice were injected with pathogens intraperitoneally and subcutaneously in the dorsal thoracic midline with 10^3* spirochetes of CB-43 strain of *B. afzelii* (500 μl of suspension per mouse) and 8 x 10^5* R. helvetica* (500 μl of suspension per mouse) overall. Mice were under anesthesia when challenged with uninfected *I. ricinus* larvae on the dorsal side at the 2nd and 14th day's post-infection allowing feeding. Four to 8 days later, the engorged tick larvae detached spontaneously from individual mice and were kept separately in vials with filter paper and closed by cloth. Vials were kept in a glass box at 23–26°C with a relative humidity of 60–80%. Random samples of 10 fully engorged larvae per mouse and random samples of 10 molted nymphs per mouse were examined for the presence of pathogens, *B. afzelii* and *R. helvetica*. An ear punch biopsy was collected from each mouse with sterile scissors and tweezers at 2 weeks post-inoculation and tested for the presence of pathogens. All mice were sacrificed by cardiac bleed followed by cervical dislocation under anesthesia after tick feeding. Subsequently, ear biopsies and organ samples were collected. All tissue samples were stored at -20°C until further investigation.

**Molecular analysis.** DNA was extracted from ticks and tissues using a DNeasy Tissue Kit (Qiagen, Valencia, CA). All samples were screened using specific primers by real-time PCR for the presence of *R. helvetica* (23S rRNA) and *B. burgdorferi* s.l. (rrlA rrlB intergenic spacer of 55-23S rDNA), respectively. The forward primer Bb23Sf (5'-CGAGTCTTAAAGGGCGATTGTAG-3') and the reverse primer Bb23Sr (5'-GCTTCAGCTGGCCATAAATAG-3') and the TaqMan probe Bb23Sp-FAM (5'-AGATGTGGTGA CCGGAAGCCGAGTG-3') were used for the detection of *B. afzelii* (Courtney et al., 2004). The reaction mixtures contained 12.5 μl of the 2x SuperHot Master Mix (Bioron, Ludwigshafen, Germany), 0.265 μl MgCl2, 1.8 μl forward and reverse primers (final concentrations of 10 pM of each primer), 0.5 μl TaqMan probe (final concentration of 10 pM), 2.775 PCR water, and 5 μl of the template in a total volume of 25 μl. Each sample was subjected to a Real-time PCR program using Bio-Rad CFX96TM Real-Time System, consisting of 3 steps: initial denaturation at 95°C for 2 min., followed by 39 cycles at 95°C for 1 s and 60°C for 1 min. For detection of *R. helvetica*, we used the forward primer Rickhelv.147f (5'-TTT GAA GGA GAC ACG GAA CAC A-3'), the reverse primer Rickhelv.211r (5'-TCC GGT ACT CAA ATC CTC ACG TA-3') and the probe Rickhelv.170p (5'-6-FAM-5'AAC GTG ACG GTA CAC TTA-TAMRA-3') (Borello et al., 2009). The real-time PCR mixtures contained 4 μl of the 5 x HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), final concentrations of 100 nM of each primer and 100 nM of the probe, and 3 μl of the template in a total volume of 20 μl. The *R. helvetica*-specific real-time PCR assay was performed using a Bio-Rad CFX96TM Real-Time System, with an initial step of 50°C for 2 min and a denaturation step at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

**Results and Discussion**

Balb/c mice were used as a host for selected pathogens in the first experiment. Organ specimens from Balb/c mice and ticks feed on mice were *R. helvetica*-negative. On the other hand, infection of Balb/c mice with *B. afzelii* was successful. It was confirmed in hearts (5/5 of mice), lungs (3/5), urinary bladders (3/5), kidney (1/5), larvae (40%; 10/25) and molted nymphs (52%; 13/25) feeding on the infected mice.

In the 2nd experiment, C3H/N mice were applied. *R. helvetica* was occasionally confirmed in ear biopsy, spleen, and urinary bladder obtained from the mice infected with both pathogens and in lung, liver, and spleen in mice infected with *R. helvetica* (Table 1). It was also found in one larva fed on *R. helvetica* infected mouse. On the other hand, *B. afzelii* was detected in ear biopsies, heart, lung, kidney, and urinary bladder tissues of infected C3H/N mice (Table 1). Larvae, which were placed on *B. afzelii* infected mice at the second day after inoculation of pathogens and molted nymphs were negative. Totally, 31% (31/100) of larvae, which were placed on these mice at 14th day after inoculation with pathogens...
and 22% (22/100) of molted nymphs were positive for the presence of *B. afzelii*.

Mice have been also used as an animal model for studying *C. burnetii* infection. In an evaluative study of 47 strains of inbred laboratory mice, 33 were found to be resistant to infection of *C. burnetii* phase I Nine Mile, 10 were partially susceptible, and 4 were susceptible (Scott et al., 1987). Viable *C. burnetii* cells were visible in organs of the sensitive mice strains. Furthermore, these mice generated protective immune response against *C. burnetii* (Scott et al., 1987). From these strains, the highest mortality (70%) was observed in the A/J strain. The BALB/c strain was reliably infected and displayed overt signs of illness, including ruffled fur and lethargy. Splenomegaly, weight loss, and seroconversion were also recorded. These characteristics could be the most reliable markers of *C. burnetii* infection in mice. In the case of C3H/HeJ strain inoculated with *C. burnetii* phase I, only the half of animals were affected (Scott et al., 1987). It indicates that mouse susceptibility to infection depends on the immune machinery of each mouse strain.

In this regards, the mouse strains that are susceptible to one bacterium are likely not sensitive to another (Bechah et al., 2008). For example, BALB/c mice represent a convenient model for epidemic typhus, but it does not constitute a model for murine typhus. On the other hand, C3H/HeJ strain inoculated with *C. burnetii* phase I, only the half of animals were affected (Scott et al., 1987). It indicates that mouse susceptibility to infection depends on the immune machinery of each mouse strain.

Table 1. *Borrelia afzelii* and *Rickettsia helvetica* positivity of tissues from C3H/N mice (No. of *B. afzelii*-positive mice / No. of *R. helvetica*-positive mice / total mice)

<table>
<thead>
<tr>
<th>Ear biopsies</th>
<th><em>B. afzelii</em>-infected mice</th>
<th><em>R. helvetica</em>-infected mice</th>
<th><em>B. afzelii</em>-<em>R. helvetica</em>-infected mice</th>
<th>Control mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5/0/5</td>
<td>0/0/5</td>
<td>5/1/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Heart</td>
<td>0/0/5</td>
<td>0/0/5</td>
<td>0/0/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Lung</td>
<td>5/0/5</td>
<td>0/0/5</td>
<td>5/0/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Liver</td>
<td>4/0/5</td>
<td>0/1/5</td>
<td>5/0/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/0/5</td>
<td>0/1/5</td>
<td>0/0/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/0/5</td>
<td>0/1/5</td>
<td>0/0/5</td>
<td>0/0/5</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>5/0/5</td>
<td>0/0/5</td>
<td>5/1/5</td>
<td>0/0/5</td>
</tr>
</tbody>
</table>

From the obtained results we can conclude, that C3H/N and BALB/c are well-accepted mice models for *B. afzelii* that are also susceptible to *C. burnetii*. On the other hand, these mice strains are unsuitable for *R. helvetica* infection at an inoculation dose of 8 x 10^4 cells. These mice strains are useless for *R. helvetica* transmission and interaction studies using *I. ricinus* as a vector. In order to dip into this problem, more extensive interaction study on vectors co-infected with
tick-borne microorganisms is necessary. Thus, the next step should include screening for suitable hosts and vectors. Besides conventional molecular biology methods, application of novel mass spectrometry imaging and electron microscopy techniques will be crucial, especially in analyzing molecular markers of infection. This knowledge may in future lead to improving prevention of these tick-borne diseases.

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