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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 surveillance, and control of Q fever in livestock is of particular importance. For this reason, Q fever was included in 2010 to the government monitoring program of animals infectious diseases also in Poland. The aim of this study was to estimate the seroprevalence of *C. burnetii* in horses and domesticated ruminants, including cattle, sheep, and goats.

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Abbreviation: CFT = complement fixation test

Materials and Methods

A total of 2,082 serum samples were collected from 124 horses, 933 cattle, 89 sheep and 936 goats between January 2016 and June 2017. Most of the tested animals showed no clinical symptoms of the disease; some individuals had a retention of the placenta or abortion episodes in the past. Sampling from horses was performed in the six herds, located in Lubelskie, Małopolskie and Podkarpackie provinces. Among tested horse breeds, the most numerous were Hucul horses (n = 50), followed by Polish Konik (n = 37), Felin Pony (n = 27), Polish Halfbred Horse (n = 5), Malopolska Horse (n = 3) and Bilgorajski Horse (n = 2). The bovine sera were collected from 358 herds situated in 14 of 16 Polish provinces, excluding Małopolskie and Lubuskie voivodeships. Moreover, 32 goat herds and 12 flocks of sheep from 10 provinces were included in serological tests. The samples were taken by authorized veterinarians during clinical studies following standard procedures

Table 1. Serological examination of cattle and small ruminants herds by ELISA

Province	Cattle				Small ruminants			
	No. of tested samples	No. of positive samples/%	No. of tested herds	No. of positive herds/%	No. of tested samples	No. of positive samples/%	No. of tested herds	No. of positive herds/%
Dolnośląskie	12	0	7	0	NA	NA	NA	NA
Kujawsko-Pomorskie	87	3/3.45	16	3/18.75	2	1/50	1	1/100
Lubelskie	77	0	25	0	4	0	2	0
Lubuskie	NA	NA	NA	NA	NA	NA	NA	NA
Łódzkie	75	3	65	3/4.62	2	0	1	0
Małopolskie	NA	NA	NA	NA	5	4 /80	2	1/50
Mazowieckie	87	1/1.15	18	1/5.56	210	1/0.48	12	1/8.33
Opolskie	87	13/14.94	40	5/12.5	NA	NA	NA	NA
Podkarpackie	93	1/1.08	93	1/1.08	34	1/2.94	3	1/33.3
Podlaskie	20	1/5	9	1/11.11	488	54/1107	8	1/12.5
Pomorskie	51	1/1.96	6	1/16.66	NA	NA	NA	NA
Śląskie	40	5/12.5	7	1/14.29	NA	NA	NA	NA
Świętokrzyskie	50	0	3	0	42	3/7.14	2	2/100
Warmińsko-Mazurskie	92	10/10.87	8	3/37.5	235	4/1.7	10	2/20
Wielkopolskie	61	0	17	0	3	3/100	3	3/100
Zachodnio-Pomorskie	101	1/0.99	44	1/2.27	NA	NA	NA	NA
total	**933**	**39/ 4.18**	**358**	**20/5.59**	**1025**	**71/6.92**	**44**	**12/27.27**

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 Tylewska-Wierzbanowska, 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 not 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.

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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 burgdorferii* 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 *B. afzelii* infection. However, they are not suitable for interaction studies with *R. 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

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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 Virology, 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 mice strains, C3H/N and Balb/c, were used. Balb/c mice were separated into 3 groups consisting of 5 animals each. The 1st and 2nd groups were inoculated with *B. afzelii* 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 10^4 *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. (rrfA rrlB intergenic spacer of 5S-23S rDNA), respectively. The forward primer Bb23Sf (5'-CGAGTCTTAAAAGGGCGATTTAGT-3'), the reverse primer Bb23Sr (5'-GCTTCAGCCTGGCCATAAATAG-3') and the TaqMan probe Bb23Sp-FAM (5'-AGATGTGGTAGA CCCGAAGCCGAGTG-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), 0.625 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 15 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 CGT AGC GTA CAC TTA-TAMRA-3') (Boretti *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

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)

	B. afzelii-infected mice	*R. helvetica*-infected mice	*B. afzelii*+*R. helvetica*-infected mice	Control mice
Ear biopsies	5/0/5	0/0/5	5/1/5	0/0/5
Blood	0/0/5	0/0/5	0/0/5	0/0/5
Heart	5/0/5	0/0/5	5/0/5	0/0/5
Lung	4/0/5	0/1/5	5/0/5	0/0/5
Liver	0/0/5	0/1/5	0/0/5	0/0/5
Spleen	0/0/5	0/1/5	0/1/5	0/0/5
Kidney	2/0/5	0/0/5	4/0/5	0/0/5
Urinary bladder	5/0/5	0/0/5	5/1/5	0/0/5

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/HeN mice are susceptible to *R. typhi*. It is also known that *R. akari, R. conorii* and *R. sibirica* are highly lethal for C3H/HeJ mice, but *R. rickettsii* and *R. australis* are not (Eisemann *et al*., 1984). *R. helvetica* did not show any pathogenic effect on Swiss mice, guinea pigs or domestic rabbits (Hajem *et al*., 2009).

Six pathogenic *Borrelia* genospecies (*B. burgdorferi* sensu stricto (s.s.), *B. afzelii, B. garinii, B. spielmanii, B. bavariensis* – originally called *B. garinii* OspA type 4 and *B. bissettii* may induce human Lyme borreliosis. Each of these genospecies is differently associated with vectors and hosts and cause distinct symptoms of the disease (Baranton *et al*., 1992; Canica *et al*., 1993; Wilske *et al*., 1996; Wang *et al*., 1997; Richter *et al*., 2006; Margos *et al.*, 2009, 2013; Hubálek *et al*., 2011). Rodents are the most important reservoir hosts for *B.afzelii*, *B. burgdorferi* s.s., *B. spielmanii* and *B. bavariensis* (Kurtenbach *et al*., 1998; Huegli *et al*., 2002; Richter *et al*., 2006). Susceptibility of laboratory mice and the course of infection depends on the genotype and age of the mice (Barthold *et al*., 1990). Sensitive strains of laboratory mice include SCID, C3H, and SWR mice (Barthold *et al*., 1990, 1999; Schaible *et al*., 1990; Zeidner *et al*., 1997). Previous studies have proven that the C57DL/6 strain, DBA/ 2J, and the BALB/c are non-sensitive to borreliae (Zeidner *et al*., 1997; Brown and Reiner, 1998; Ma *et al*., 1998; Brown *et al*., 2003; Ganapamo *et al*., 2003). However, recent studies consider BALB/c mice susceptible to *B. afzelii* infection (Tonetti *et al*., 2015; Jacquet *et al*., 2016).

Another significant factor determining the severity of the disease in humans and animals is pathogenicity of the infecting bacterial strain and potential co-infection with other tick-borne pathogens that may affect the transmission of the pathogen to the vectors and hosts (Nadelman *et al*., 1997; Schwartz *et al*., 1997; Thomas *et al*., 2001; Jacquet *et al*., 2016). However, the relationships of microorganisms inside the ticks and their potential role in the transmission of disease are not entirely understood. Alterations in transmission between two strains of *B. afzelii* in a vector-host experiment using BalB/cByJ mice were already recorded with distinct courses of infection (Jacquet *et al*., 2016). Thus, to assess the impact of co-infection on the rate of transmission between the vectors themselves or the vector and its host, we examined the interactions of two tick-borne pathogens, *R. helvetica* and *B. afzelii*.

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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