JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

STUDIES ON THE OCCURRENCE OF *COXIELLA BURNETII* INFECTION IN TICKS IN SELECTED EASTERN AND CENTRAL REGIONS OF POLAND

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doi: 10.15414/jmbfs.2016.5.4.355-357

ARTICLE INFO

Received 1. 12. 2014
Revised 31. 3. 2015
Accepted 12. 11. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

Arthropods may play a role in the transmission of *Coxiella burnetii* as they transmit pathogen both mechanically (as flies), and actively during the host blood suction. The aim of this study was to identify the *C. burnetii* occurring in ticks harvested from both domestic and livestock animals, as well as from wildlife in randomly selected regions of Poland. The total number 1126 ticks was collected in four regions of Poland: the Masovian, Lublin, Łódź and Podkarpackie Provinces. Among ticks collected from May 2011 to August 2012, the presence of *IS1111* gene sequence was detected in 15 (1.33%) cases by real-time PCR test. Among the ticks harvested from domestic and livestock animals in 12 cases (3.1%), and in case of ticks found in open-space rodents in 3 cases (0.4%) we found the presence of marker sequences. The low percentage of *C. burnetii* infected ticks indicate a limited role of these arthropods in Q fever transmission.

Keywords: *Coxiella burnetii*, ticks, *IS1111*, real-time PCR

INTRODUCTION

In humans, the most frequent routes of infection are via inhalation (**Dutkiewicz *et al.*, 2011**), direct contact of pathogen with damaged skin and mucous membrane, via the alimentary tract, and as a result of contact with *C. burnetii* infected ticks. In the latter case the infection can be transmitted during, the tick blood suction as well as by contact with its excrements. In the *Dermacentor andersoni* faeces the bacteria survive even up to 580 days (**Woldehiwet, 2004**).
The reservoir of *C. burnetii* comprises a wide range of species, including all vertebrates, as well as parasitic invertebrate animals. Reported data described cases of *C. burnetii* isolation from samples of animals such as sheep, goats, cattle, dogs, cats and many species of wild-living vertebrates, including bears, bison, deer, boars, rabbits, hares, shrews and marsupials. Arthropods may play a significant role in pathogen transmission, as they transmit *C. burnetii* both mechanically (e.g. flies), and actively during the host blood suction, the host may be also infected through the cutaneous or inhalation contact with the parasites faeces (**Marrie, 1990; Anusz, 1995**). The role of ticks in spreading the pathogen results from theirs manner of feeding and the ability of transstadial transmission. The ability of pathogen transmission (including transovarial) has been reported in about 40 ticks species (**Mediannikov *et al.*, 2010**). Since ticks can reside on at least two to three different hosts (mainly mammals) during their life-cycle, they are considered as one of the factors determining the persistence of *C. burnetii* in the environment (**Fard and Khalili, 2011**). The infection of ticks by *C. burnetii* occurs during the blood sucking of on infected mammal or other vertebrate, whereby they become a vector of this microorganism (**Norlander, 2000**; **Bossi *et al.*, 2004; Hartzell *et al.*, 2008; Oyston and Davies, 2011**).
The aim of this study was to identify the *C. burnetii* occurring in ticks harvested from both domestic and livestock animals, as well as wildlife in randomly selected regions of Poland.

MATERIALS AND METHODS

Ticks collection

Ticks were collected in four regions of Poland: the Masovian Province (central region of Poland) - 27 *Ixodes ricinus*: 5 from wildlife (deer, boar), 2 from domestic animals (cats, dogs), 20 from cattle; 4 *Dermacentor reticulatus* from domestic animals, Lublin Province (eastern Poland) - 95 *I. ricinus*: 57 from wildlife, 18 from open-space rodents, 8 from cattle, 12 from domestic animals, 2 *D. reticulatus* from domestic animals, 16 *Ixodes hexagonus* from domestic animals, Łodź Province (central Poland) - 18 *I. ricinus* from domestic animals; 2 *I. hexagonus* from domestic animals, and Podkarpackie Province (south-eastern Poland, mountains and sub-mountains region) - 699 *I. ricinus*: 325 from wildlife, 75 from open-space rodents, 62 from domestic animals, 184 from cattle, 53 from goats; 5 *I. persulcatus* from wildlife; 257 *D. reticulatus* from wildlife; 1 *Dermacentor marginatus* from wildlife.
The specimens were collected from May 2011 to August 2012. A total of 1126 ticks were collected, representing the following species: *I. ricinus* (74.5%), *D. reticulatus* (23.3%), *I. persulcatus* (0.45%), *I. hexagonus* (1.66%) and *D. marginatus* (0.09%).
Ticks were removed directly from animals, 743 specimens were taken from wild animals and another 383 ticks were harvested from domestic and livestock animals.
Among the *I. ricinus* species 839 mature individuals were collected, including 707 females feeding, 120 males and 12 nymphs. From the *D. reticulatus* species 117 adult females and 146 males were caught, and also 5 females from the species *I. persulcatus*, 17 adult females and one nymph from the *I. hexagonus* species, and from the *D. marginatus* species - one adult female. Ticks were placed in sterile 2 ml tubes and stored at -20°C until further analysis.

Ticks identification and treatment, Genetic material isolation

Identification of tick species was carried out according to the identification key (**Siuda, 1993**) using a dissecting microscope at magnification range 3.5 - 90 × in the reflected artificial light, and with regard to the larvae in transmitted light.
For tick treatment, the modified method described by **Mediannikov *et al.* (2010)** was implemented. In order to eliminate the possible microbiological contamination including *Bacillus cereus* group, ticks were treated with 0.5% solution of sodium hypochlorite for 10 minutes. In the next stage, the ticks were rinsed with distilled water, dried and re-suspended in 70% ethanol for 10 minutes. Finally, the samples were rinsed with distilled water and PBS. Remains of the fluids were then removed and dry ticks were re-suspended in 0.6 ml of MEM (Minimum Essential Medium, Sigma-Aldrich, USA) without antibiotic and homogenized by Stuart®SHM-1 apparatus. The homogenate was filled up to 2 ml

with MEM. Chitin remains were precipitated and liquid layer (1 ml) was centrifuged at 25 000 × g, the pellet was re-suspended in MEM (5 ml) and then twice filtered through 0.45 µm membrane. The filtrate was used for infection the BGM cell line (African monkey green kidney, HPA Culture Collection, UK) (**Mediannikov *et al*., 2010)**. Bacterial DNA was isolated from 1 ml of the suspension obtained after homogenization of the ticks, using Genomic AX Tissue Mini Spin Kit (A&A Biotechnology, Gdynia, Poland) according to manufacturer's recommendations.

Real-time PCR

Real-time PCR method was used for detecting the presence of insertion sequence *IS1111* (transposase gene, GenBank: M80806) (**Klee *et al*., 2006**) using the following primers and probe sequence: *IS1111F*: 5'-GTCTTAAGGTGGGCTGCGTG-3', *IS1111R*: 5'-CCCCGAATCTCATTGATCAGC-3', *IS1111P*: 5'- FAM – AGCGAACCATTGGTATCGGACGTTT-TAMRA-TATGG-Pho-3'.
The real-time PCR reaction was performed in a capillary system in the LightCycler 2.0 (Roche, Poland) in a final reaction volume of 20 µl. The reaction mixture consisted of the LightCycler TaqMan Master Kit (Roche, Germany) - 10 × conc., primers - 0.5 µM, probe - 0.15 µM and 5µl of template DNA.
The reaction using hydrolyzing probes for *IS1111* gene sequence was carried out according to previously optimized thermal profile: 10 min. at 95°C (initial denaturation), 15 s at 95°C, 30 s at 60°C (40 cycles of amplification), 30 s at 40°C.
As a positive control DNA extracted from *C. burnetii* strain Nine Mile phase I was used, and as a negative control a reaction mixture without template DNA was used. Real-time PCR was performed for 40 cycles, and the test samples for which C_t (Cycle threshold) was lower than 36 were considered as positive. For samples for which the fluorescence signal was weak (>36 C_T), additionally a second round of real - time PCR using the same set of primers and probes was performed.

***C. burnetii* culturing**

Culturing of the BGM cell line was carried out in the presence of MEM culture medium containing: 2 mM glutamine (Sigma-Aldrich, USA), 1% Non-essential amino acids (NEAA, (Sigma-Aldrich, USA)), 10% FBS (Fetal bovine serum, Sigma-Aldrich, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, USA) at 37°C in the presence of 5% CO_2.
The filtrate obtained from homogenized ticks in the amount of 0.5 ml was used for infection of the BGM cell line. Culturing of the infected cell line was carried out at 37°C in the presence of 5% CO_2. No antibiotics were added to culture medium. *C. burnetii* was then recovered from the cell line, in order to do that the method using digitonin described by **Cockrell *et al*. (2008)** was employed.

RESULTS AND DISCUSSION

From 1126 ticks harvested in the selected regions of Lublin, Podkarpackie, Masovian, and Łódź Provinces, the presence of *C. burnetii*-specific *IS1111* gene sequence was found in 15 (1.33%) cases **(Tab. 1).** Among the positive samples, five were acquired in Lublin Province (4 – *I. ricinus*, 1 – *D. reticulatus*), seven in Masovian and Łodź Provinces (6 – *I. ricinus*, 1 – *I. hexagonus*) and three in Podkarpackie Province (*I. ricinus*). In case of seven tick samples, the C_T fluorescence had the value of 31, for the next two it was above 35 cycles, and for others the signal was detected in the second round of real-time PCR. Among ticks harvested from domestic and livestock animals in 12 cases (3.1%), and in case of ticks found in open-space rodents in 3 cases (0.4%) we found the presence of marker sequences.

Table 1 Summary of positive test results for the presence of *C. burnetii* infections in ticks

No.	Province	Place/District	Animal species/ source	Tick species	Description
1.	Lublin	Puławy	Dog	*I. ricinus*	A, P, F
2.			Dog	*I. ricinus*	A, P, F
3.			Cat	*I. ricinus*	A, P, F
4.			Dog	*I. ricinus*	A, P, F
5.	Lublin	Ryki	Dog	*D. reticulatus*	A, G, F
6.	Masovian	Pułtusk	Cat	*I. ricinus*	A, P, F
7.			Dog	*I. ricinus*	A, P, F
8.			Dog	*I. ricinus*	A, P, F
9.			Dog	*I. ricinus*	A, P, F
10.	Łódź	Łódź	Cat	*I. ricinus*	A, P, F
11.			Dog	*I. ricinus*	A, P, F
12.			Dog	*I. hexagonus*	A, P, F
13.	Podkarpackie	Sanok	Open-space rodent[1]	*I. ricinus*	A, P, F
14.			Open-space rodent[1]	*I. ricinus*	A, P, F
15.			Open-space rodent[2]	*I. ricinus*	A, G, F

[1] – Striped field mouse (*Apodemus agrarius*), [2] – common vole (*Microtus arvalis*), A – Adult individual, P – The individual was feeding, G – The individual was not feeding, F – Female

Additionally, the biological material isolated from ticks and cultured in BGM cell line gave positive results of amplification in two cases of *I. ricinus:* from Masovian and Lublin Provinces.
The surveillance among ticks is rarely conducted in Poland. The studies initiated by **Anusz (1990)** revealed *C. burnetii* in females *I. ricinus* (Warmian-Masurian Province, north-eastern Poland). Simultaneously **Tylewska-Wierzbanowska *et al.* (1996)** revealed 3 (0.19%) *C. burnetii* positive ticks, out of 1580 collected in various regions of Poland (former Bydgoszcz, Koszalin, Piła, Płock, Warszawa, Kielce and Tarnobrzeg Provinces). Contrarily, another group (**Niemczuk *et al*., 2011**) carried out studies between 2009 and 2010 on a limited number of ticks in Lublin Province and yielded 33.3% positive results of *C. burnetii* genetic material. Moreover, the same researchers between 2011 – 2013 examined the *I. ricinus* collected from endemic area in south-eastern Poland. The percentage of ticks infected with *C. burnetii* was 15.9% (**Szymańska-Czerwińska *et al.*, 2013**). Supposedly, such high percentage of positive results is related to sampling the specimens from natural focus of Q fever. Studies on the presence of *C. burnetii* among ticks from other parts of the world revealed rather low percentage of infected individuals (from 0.3% to 7.7%) (**Mediannikov *et al*., 2010; Fard and Khalili, 2011; Špitalská *et al*., 2003; Toledo *et al*., 2009; Hildebrandt *et al*., 2011; Sprong *et al*., 2012**).
In our studies only two isolates were recovered from all 15 BGM cell line cultures. Due to intracellular type of *C. burnetii* infection, the method of bacteria recovery is crucial – significant losses of material may be sustained during the isolation procedure. The digitonin, which has high affinity to PV (parasitophorous vacuole, containing cholesterol) was successfully utilized in the present study. This intracellular structure is the place of *C. burnetii* proliferation. On the other hand, bacterial coinfections (such as *Borrelia* spp.), noted mainly among samples obtained from ticks, disturbed some cell cultures and impaired *C. burnetii* recovery from the cells. Sonication method, which is an alternative option for *C. burnetii* isolation, was not used due to accompanying risk related to unintentional generation of biological aerosol (**Cockrell *et al*., 2008**). We used multi-copy *IS1111* gene (7 to 110 per one cell of *C. burnetii)*, which is a very convenient and sensitive genetic marker as a tool for *C. burnetii* screening in ticks (**Klee *et al*., 2006**; **Bielawska-Drózd *et al*., 2013**).
Pathogen may be transmitted by number of ticks species, the castor bean tick (*I. ricinus*) seems to be the most epidemiologically important species in Poland (**Tylewska-Wierzbanowska *et al*., 1996**; **Bielawska-Drózd *et al*., 2013**). Not

only it is the most prevalent species, but also it has a significant range of hosts. This assumption was confirmed in the current study, showing that the most of infected ticks were *I. ricinus*. The meadow tick (*D. reticulatus*) may be taken into account as the second most predominant tick in the eastern Poland (**Tylewska-Wierzbanowska and Chmielewski, 2010**).

Currently, the role of ticks as vectors and reservoir of Q fever is disputable. Some researchers (**Mediannikov *et al*., 2010; Tylewska-Wierzbanowska and Chmielewski, 2010; Sprong *et al*., 2012**) claim that ticks, as vectors of *C. burnetii* do not play a significant role in disease transmission among animals and humans. Contrarily, **Toledo *et al*. (2009)** observed high number of infected individuals among *H. lusitanicum* and *D. marginatus* population, which support the hypothesis about their role in Q fever transmission. Also other authors claim that ornate sheep tick (*D. marginatus*) is the most significant vector of *C. burnetii* in the middle Europe (**Hildebrandt *et al*., 2011**). Results of the current study demonstrated a low proportion of infected ticks (1.33%), which may confirm the first thesis. Nonetheless, ticks may be an important factor in *C. burnetii* transmission among wild animals, such as rodents, lagomorphs or wild birds (**Fard and Khalili, 2011; Astobiza *et al*., 2011**).

CONCLUSION

In our research, a low percentage of *C. burnetii* infected ticks (1.33%) indicated by the probe specific real-time PCR for multi-copy insertion sequence (*IS1111*), reveals limited role of these arthropods in Q fever transmission in Poland.

Acknowledgments: The study has been funded by the Ministry of Science and Higher Education of the Republic of Poland (Grant No. N N308 562540). Reference strains of *C. burnetii* (F4, Nine Mile, RT-1 and Z349-36/94) were kindly provided by Dr. Dimitrios Frangoulidis and Dr. Holger Scholz form Microbiology Institute of German Armed Forces (Munich, Germany).
We also would like to acknowledge laboratory technicians Barbara Ciecierska and Dorota Sikorska for the technical assistance in the samples preparation.

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