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Prevalence of *Coxiella burnetii* infection in foresters and ticks in south-eastern Poland and comparison of diagnostic methods

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Abstract

Introduction: *Coxiella burnetii* is an aetiologic agent of Q fever. Ticks have been considered as a reservoir and a vector of *C. burnetii*. The outbreaks of Q fever were noted in different parts of Europe both in animals and humans. Q fever is zoonotic diseases characterized by sudden fever, headache, atypical pneumonia and abortions.

Aim: The aim of the study was to investigate the presence of *C. burnetii* infection in foresters in the south–eastern endemic region of Poland and comparison of diagnostic methods.

Materials and methods: The serum samples were collected from 304 humans (foresters) from the 12 superintendency in the south-eastern Poland. Additionally, 1,200 ticks *Ixodes ricinus* were collected from the same area. The sera samples were tested by using serological methods (CFT, ELISA and IFA) and the ticks were tested with the use of real-time PCR.

Results: The results demonstrates that infections with *C. burnetii* are present in foresters in the south-eastern Poland. The serological results showed both chronic and acute form of Q fever. The statistical analysis of the positive results obtained by three different serological methods revealed that values of $\chi 2$ were statistically significant between IFA and ELISA and CFT and IFA. The final percentage of examined foresters classified as positive was 10.52%. No significant differences were observed between CFT and ELISA ($p=0.25$).The percentage of infected ticks was 15.9%.

Conclusion: The study demonstrates that infections with *C. burnetii* are present in foresters in the south-eastern Poland. The comparison of serological methods showed that their diagnostic potential is differentiated but the most useful tool is IFA.

Key words

Coxiella burnetii, Q fever, foresters, serologic diagnostics

INTRODUCTION AND OBJECTIVE

Coxiella burnetii is an etiologic agent of Q fever. The reservoir of this bacterium is extensive although only partially known and includes mammals, birds, and arthropods, mainly ticks. Ticks have been considered as a reservoir and a vector of *C. burnetii*. The infection is transmitted from one stage to the next in the tick's cycle, and transovarian infection is observed. Q fever is a zoonotic disease characterized by sudden fever, headache, atypical pneumonia and abortions. Human infections are mostly related to infected ruminants, e.g. sheep, goats and cows. *C. burnetii* is an obligatory intracellular parasite, extremely infectious and weather resistant, and may survive for several weeks in the environment [1].

Outbreaks of Q fever have been noted in different parts of Europe, both in animals and humans. The largest Q fever outbreaks occurred in the Netherlands in 2007 and in Germany in 2003 and 2005 [2]. The first outbreak of Q fever in Poland was recorded in 1956 [3]. Between 2005 and 2010, a few Q fever outbreaks were been recognized in southern Poland [4], a region of the country known as an endemic area for *C. burnetii*, as confirmed by preliminary studies of ticks in endemic area [5]. The diagnosis of Q fever in humans is based mainly on serological methods, such as indirect immunofluorescence assay (IFA), complement fixation test (CFT), or ELISA [6]. Molecular assays, such as PCR and real-time PCR are used as confirmatory methods [7, 8]. Q fever in humans is diagnosed principally by serology and antibody patterns which differentiate between acute, convalescent, and chronic Q fever. The smooth, full-length polysaccharide (phase I, virulent) form of *C. burnetii* occurs in nature and is maintained in laboratories by passage through animal hosts [7]. Antibodies against this form of *C. burnetii* are predominantly observed in chronic Q fever. Following several passages by cell culture, *C. burnetii* undergoes a phase of transition known as phase II. Antibodies against this form are the first to be detected in acute Q fever patients [8].

The aim of the presented study was to investigate the presence of *C. burnetii* infection in foresters in the south–eastern endemic region of Poland, and comparison of diagnostic methods.

MATERIALS AND METHODS

The serum samples were collected from 304 humans occupational exposed (foresters) to infection of *C. burnetii* from the 12 superintendency in south-eastern Poland. Tested

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samples were randomly collected, and foresters selected for the study did not show clinical signs of Q fever.

1,200 of *Ixodes ricinus* species were collected between 2011 – 2013 from south-eastern Poland and tested by using PCR. The detailed information about the place of harvesting the ticks are presented in Table 1.

The enzyme-linked immunosorbent assay – ELISA (Nova Tec Immundiagnostica GmbH, NovaLisa) for the qualitative determination of IgG class antibodies against *C. burnetii* phases 1 and 2 and IgM class antibodies against *C. burnetii* phase 1 was performed according to the manufacturer's instructions. The presence of IgM class antibodies phase 2 (2–3 weeks after infection) and IgG class phase 2 (2 months after infection) confirmed the acute phase Q fever, while in chronic infection the IgG phase 1 are detected. Interpretation of the results was based on the value of NTU (nephelometric turbidity unit). Sera were considered to be ELISA negative if NTU < 9, dubious if 9 ≥ NTU ≤11, positive >11.

The immunofluorescence antibody assay (IFA) was performed by using Focus Diagnostic Cypress, California 906030, USA. The interpretation of results was based on the presence of antibodies class. If the reactivity of antibodies to both phase I and II antigens (titre≥ 16) was observed, it strongly pointed to *C. burnetii* infection. Phase I antibody titers of greater than or equal to phase II antibodies titers were consistent with chronic infection or convalescent phase of Q fever. If the reactivity of antibodies to both phase I and II antigens (titre <16) was noted, the result was negative. The titers of antibodies phase II > than phase I characterized the form of Q fever. The reactivity to phase II antigen only (titres < 256) argued against *C. burnetii* infection. The IFA is a reference method for detection of infection with *C. Burnetii;* therefore, the final classification of results was based on the IFA results.

For the complement fixation test (CFT), Institut Virion/ Serion GmbH (Germany) and the Institute of Biotechnology, Sera and Vaccines Biomed SA reagents were used. The starting dilution of the examined sample was 1:5, the ending dilution was 1:80. Serum was considered as positive when a partial inhibition of haemolysis (++) was observed in the dilution 1:10. Two different antigens of Coxiella burnetii specific for phase I and II, were used.

The genomic DNA from ticks was extracted using a commercial kit (QIAamp DNA mini kit, Qiagen), according to the manufacturer's protocol. The ticks were homogenized before extraction of DNA. Real-time PCR assays targeting the IS*1111* gene of *C. burnetii* described previously by Panning were performed [8].

A 5 µl aliquot of extracted DNA was subjected to 20 microlitres of real-time reaction mixture contained: 2.5 µl of 10 x PCR buffer, 2.0 µl of 50mM $MgCl_2$, 2.0 µl of 10 mMdNTP, 2.0 µl of 10 pmol primer CoxbS (GTAGCCCGATAAGCATCAAC), 2.0 µl of 10 pmol primer CoxAs, 0.75 µl probe (FAM-TCATCAAGGCACCAATGGTGGCCA-TAMRA) 8.5 µl sterile water and 0.25 µl of 5 U/µl thermostable polymerase DNA. Cycling conditions in the 7500 Fast real-time PCR system (Applied Biosystems) were: 94 °C/2 min, and 45 cycles of 94 °C/15 sec, 60 °C/30 sec. The positive control of *C. burnetii* DNA (Genecam, Germany) and negative control were used in real-time PCR reaction.

Pearson's chi-square tests were used to compare the results obtained using 3 methods: CFT, IFA and ELISA. Furthermore, correlation coefficients were calculated between results obtained for IgG class antibodies for both phases of Q fever (I and II). All analyses were conducted with the use of statistical software StatSoft, Inc., 2011, STATISTICA (data analysis software system), version 10. For the purpose of the study, the following guidelines for interpreting positive correlations were used: r=0 – 0.09: no or negligible relationship, r=0.1 – 0.29: weak relationship; r=0.3 – 0.49: moderate relationship; r=0.5 – 0.69: strong relationship; r= 0.7 – 0.99 – very strong relationship; r=1: full relationship. The established criteria were only crude estimates for interpreting strengths of correlations. For the purpose of the proper estimation of correlations, doubtful results in ELISA were excluded from the calculations.

Additionally, the $\chi 2$ (chi-square) tests with appropriate corrections at the level of significance α=0.05 were used for statistical analysis to compare the final positive results obtained by 3 different serological methods.

Table 1. Summary of investigations in foresters and ticks.

Place of work	District	No. of examined sera	No. of positive sera in CFT	No. of positive sera in IFA	No. of positive sera in ELISA	No. of tested ticks	No. of positive ticks in PCR
Józefów	Lublin	20	0	1 (5.0%)	0	80	26 (32.50%)
Mircze	Lublin	25	1 (4.0%)	1 (4.0%)	0	110	25 (22.73%)
Radzyń Podlaski (first circuit)	Lublin	25	0	4 (16.0%)	0	67	14 (20.89%)
Krasnystaw	Lublin	20	0	1 (5.0%)	0	89	18 (20.22%)
Janów Lubelski	Lublin	31	1 (3.22%)	4 (12.90%)	1 (3.22%)	120	9 (7.50%)
Augustów	Podlaski	30	1 (3.33%)	2 (6.66%)	0	89	15 (16.85%)
Sarnaki	Mazowsze	22	0	2 (9.09%)	0	110	10 (9.09%)
Radzyń Podlaski (second circuit)	Lublin	26	1 (3.85%)	0	0	93	9 (9.67%)
Nowa Dęba	Podcarpathia Podcarpathia	25	2 (8.0%)	3 (12.0%)	0	112	11 (9.82%)
Lesko	Podcarpathia	25	0	0	0	122	19 (15.57%)
Stary Sącz	Małopolska	25	0	2 (8.0%)	0	110	20 (18.18%)
Olkusz	Małopolska	30	3 (10.0%)	12(40.00%)	6 (20%)	98	15 (15.31%)
Total	-	304	9 (2.96%)	32 (10.52%)	7 (2.3%)	1,200	191 (15.92%)

RESULTS

The results of serological assays (CFT, IFA and ELISA) are presented in Tables 1 and 2. Table 1 presents the number of results depending on the localization of sampling. Table 2 presents detailed information about results obtained by using the 3 different serological methods. The results show that antibodies anti-*C. burnetii* were present in foresters from the tested superintendencies, with the exception of the second circuit in Radzyń Podlaski and Lesko. The percentage of seropositive samples was differentiated depending on the methods used. The highest percentage of seropositive samples was noted when the IFA was used. The highest percentage of seropositive foresters was noted in Olkusz (40.00%) and the first circuit in Radzyń Podlaski (16.0%), while the lowest in Mircze (4.0%).

The percentage of positive samples of tested sera characteristic for the chronic form of Q fever in the IFA was 10.52%; whereas ELISA and CFT confirmed the chronic form of infection only in 1.31% and 2.3% of tested samples, respectively. The IFA did not detect the presence of the acute form of *C. burnetii* infection. The acute form of the disease was detected in 2 samples in CFT and one in ELISA; however, the IFA did not confirm these results as the acute, but as the chronic form. Two sample were doubtful for the acute form in ELISA. In 2 out of 304 tested samples by ELISA, the identification of the infection form (acute or chronic) was not possible; however, the IFA confirmed the chronic form in one serum. One doubtful result in ELISA without the possibility of the identification the form of disease was obtained; however, the IFA confirmed that it was a chronic infection.

Table 2. Detailed results of serological assays in foresters.

sample	CFT			IFA			ELISA				Final classification on the basis of 3 serological tests
	IgG, IgM I	IgG, IgM II	final result	IgG I titre	IgG II titre	final result	IgM F2 units	IgG F2 units	IgG F1 units	final result	
1.	negative	negative	-	16	16	positive chronic	9	3	4	doubtful acute	positive chronic
2.	negative	negative	-	16	-	negative	3	3	7	negative	Negative
3.	negative	positive	Positive acute	16	16	positive chronic	7	5	8	negative	positive chronic
4.	negative	negative	-	16	16	positive chronic	6	3	7	negative	positive chronic
5.	negative	negative	-	16	16	positive chronic	5	3	6	negative	positive chronic
6.	negative	negative	-	16	-	negative	5	3	5	negative	Negative
7.	negative	negative	-	-	16	negative	3	1	5	negative	Negative
8.	negative	negative	-	16	16	positive chronic	1	4	7	negative	positive chronic
9.	negative	negative	-	16	16	positive chronic	6	4	8	negative	positive chronic
10.	negative	negative	-	16	16	positive chronic	6	4	5	negative	positive chronic
11.	negative	negative	-	-	16	negative	5	1	3	negative	Negative
12.	negative	negative	-	32	32	positive chronic	2	8	7	negative	positive chronic
13.	negative	negative	-	16	16	positive chronic	4	19	15	positive chronic/ acute	positive chronic
14.	negative	negative	-	16	16	positive chronic	5	8	14	positive chronic	positive chronic
15.	negative	negative	-	16	16	positive chronic	4	5	13	positive chronic	positive chronic
16.	positive	positive	positive chronic	128	128	positive chronic	6	8	9	doubtful chronic	positive chronic
17.	negative	negative	-	-	-	negative	8	4	11	doubtful chronic	Negative
18.	negative	negative	-	16	-	negative	8	2	8	negative	Negative
19.	negative	negative	-	16	16	positive chronic	10	13	7	positive acute	positive chronic
20.	negative	negative	-	-	16	negative	7	4	19	positive chronic	Negative
21.	negative	negative	-	16	16	positive chronic	9	4	10	doubtful chronic	positive chronic
22.	negative	negative	-	64	64	positive chronic	12	15	26	positive chronic/acute	positive chronic

Table 2 (Continuation). Detailed results of serological assays in foresters.

sample	CFT			IFA			ELISA				Final classification on the basis of 3 serological tests
	IgG, IgM I	IgG, IgM II	final result	IgG I titre	IgG II titre	final result	IgM F2 units	IgG F2 units	IgG F1 units	final result	
23.	negative	negative	-	16	-	negative	5	3	4	negative	Negative
24.	positive	negative	positive chronic	16	16	positive chronic	5	4	5	negative	positive chronic
25.	negative	negative	-	16	16	positive chronic	5	5	16	positive chronic	positive chronic
26.	negative	negative	-	16	16	positive chronic	3	3	4	negative	positive chronic
27.	negative	negative	-	16	16	positive chronic	5	3	6	negative	positive chronic
28.	positive	positive	positive chronic	16	16	positive chronic	7	3	4	negative	positive chronic
29.	negative	negative	-	16	16	positive chronic	8	3	7	negative	positive chronic
30.	negative	negative	-	16	16	positive chronic	4	1	3	negative	positive chronic
31.	negative	negative	-	16	16	positive chronic	8	3	6	negative	positive chronic
32.	positive	negative	positive chronic	16	-	negative	3	1	7	negative	Negative
33.	positive	negative	positive chronic	16	16	positive chronic	11	5	8	doubtful acute	positive chronic
34.	positive	negative	positive chronic	16	16	positive chronic	9	2	3	doubtful acute	positive chronic
35.	negative	negative	-	32	16	positive chronic	5	4	8	negative	positive chronic
36.	negative	negative	-	16	16	positive chronic	9	4	11	doubtful acute/chronic	positive chronic
37.	negative	negative	-	16	16	positive chronic	5	3	6	negative	positive chronic
38.	negative	negative	-	16	16	positive chronic	5	3	6	negative	positive chronic
39.	negative	negative	-	16	16	positive chronic	8	2	4	negative	positive chronic
40.	positive	negative	positive chronic	16	16	positive chronic	2	1	3	negative	positive chronic
41.	negative	positive	positive acute	16	16	positive chronic	5	1	3	negative	positive chronic
42–304	negative	negative	-	-	-	negative	<5	<5	<5	negative	Negative

Statistical analysis of the differences between final positive results obtained by 3 different serological methods revealed that values of $\chi 2$ with appropriate correction were statistically significant between IFA and ELISA and CFT and IFA. No significant differences were observed between CFT and ELISA ($p=0.25$). The χ^2 test confirmed that in most cases the results obtained by means of different compared methods were correlated with each other ($P<0.05$); the correlations, however, had different strengths. Table 3 shows the values of the correlation coefficient (r).

On the basis of established criteria, all the results were classified to the group of 'weak and average relationship'. The results obtained by ELISA and CFT for both phases of Q fever were not correlated. No correlation of moderate relationship was observed when comparing ELISA with CFT for both chronic (I) and acute Q fever.

Table 3. Results of statistical analysis for compared methods.

Method	CFT I phase	CFT II phase	IFA IgG I phase	IFA IgG II phase	ELISA IgG I phase	ELISA IgG II phase	ELISA IgM I phase
	r coefficient						–
CFT phase I	1	–	0.4	–	0.04*	–	–
CFT phase II	–	1	–	0.31	–	–0.01*	–
IFA IgGI	0.4	–	1	–	0.39	–	–
IFA IgGII	0.31	–	–	1	–	0.45	–
ELISA IgG I	0.04*	–	0.39	–	1	–	–
ELISA IgG II	–	–0.01*	–	0.27	–	1	
ELISA IgGM	–	–	–	–	–	–	–

* statistically insignificant correlation

The results of PCR are presented in Table 1 and show that the percentage of ticks infected with *C. burnetii* was 15.9%. The highest percentage of infected ticks was noted in Józefów and Mircze.

DISCUSSION

Human infection with *C. burnetii* has been reported from many countries, including Poland [2, 5, 9], where the first outbreak of Q fever was diagnosed in 1956 [3]. Since then, a few outbreaks of the disease, both in humans and animals, have been recorded in the country [5, 10]. The largest outbreak of Q fever was reported in Ulhówek (Hrubieszowski Province, formerly the Zamość Province) in 1982 when an outbreak of Q fever was detected in cattle. Then seroconversion and clinical symptoms were noted in people who worked on a farm and in their family members. More than 1,300 people fell ill, and the epidemic involved the area of Hrubieszów and Tomaszów Lubelski districts [11]. 30 years later, Chmielewski et al. conducted a phylogenetic analysis of *C. burnetii* strains isolated during the epidemic of Q fever in Ulhówek and environs [12]. The natural focus of *C. burnetii* in this area continues and the results of our investigations provide evidence for the presence *C. burnetii,* both in humans and ticks.

Over the years, the Lublin Province has been the area of investigations of Q fever, both in humans and animals [13, 14, 15, 16]. The serolological studies performed by Cisak et al. among the farmers living in villages located in Lublin Province confirmed the presence of antibodies to *C. burnetii* phase I antigen [17]. These results suggest that in the past the examined agriculture region could have been an epidemic area of Q fever. The literature data evidences that the south-eastern region of Poland is considered to be an endemic area for the occurrence of *C. burnetii*. The outbreak of Q fever in cattle connected with transmission of infection to humans was described in Tarnogród and Dębno in 2008. The results of these investigations were preliminary described [11]. The investigations included foresters occupationally exposed to contact with ticks and animals. The presented study of foresters and ticks provide further evidence for the natural focus of *C. burnetii* in this region. The investigations confirmed both the seroconversions in foresters and presence of the specific sequences of DNA for *C. burnetii* in tested ticks. The reference methods for humans (IFA) confirmed the presence of the chronic form of *C. burnetii* infections in 9.86% of tested foresters. In available literature, the chronic form of Q fever in humans in Poland has also been described previously [14, 16]. According to the literature data, chronic Q fever may develop in 1–5% of patients many months and even the years after initial infection [18, 19]. No clinical manifestations of the chronic form have been observed; however, the most frequent symptom is endocarditis. Due to the fact that the chronic form of Q fever can cause chronic endocarditis, effective diagnosis of Q fever in humans exposed to contact with the pathogen is crucial. The tested foresters selected for investigations were exposed to contact with ticks and animals, either or both of which could have been the sources of infection.

The investigations of ticks (*Ixodes ricinus*) taken from the endemic area of Q fever confirmed the presence of *C. burnetii.* The percentage of positive ticks in our investigations was 15.9%. Previous studies of ticks collected from places in the endemic region of Poland where local outbreaks of Q fever occurred, also confirmed the pathogen in these arthropods [5].

A similar evaluation of the prevalence of *C. burnetii* in ticks was performed in the Netherlands after a large outbreak of Q fever. However, the prevalence of the pathogen in questing ticks was lower than in the conducted studies (0.2%) [20]. Detection of the questing *C. burnetii*-positive *I. ricinus* nymphs and adult ticks have been carried out previously in Hungary and Slovakia [21, 22, 23, 24], but not in Spain [25, 26] or Luxemburg [27]. In Germany, negative results were reported in the past [28], but recent study have reported that 1.2–3.0% of the ticks were found to be positive [29]. Detection of *IS 1111* gen in ticks may suggest that ticks can be an important vector for *C. burnetii* in the endemic region of Poland. Ticks acquire *C. burnetii* during blood meal on infected animals and could transmit the bacterium to the other mammals during the next blood meal, or by aerogenic spread of dried tick faecal excretions, maintaining *C. burnetii* in wildlife. Previous studies of ticks collected from places in endemic region of Poland where local outbreaks of Q fever occurred, confirmed the presence of pathogen in this arthropods [5]. In the literature data, there is no information about prevalence of *C. burnetii* in wild animals in the endemic area of Q fever in Poland; therefore, it is difficult to evaluate properly whether or not the animals are the sources of infection in the tested foresters. Q fever diagnosis based on clinical symptoms or post-mortem picture is very difficult and almost impossible as signs and symptoms of the disease are not specific [30]. In particular, clinical symptoms of infected animals do not occur. The infection of *C. burnetii* can be detected by serological methods, such as the IFA, ELISA and CFT.

Statistical analysis of the presented results (value of Pearson's correlation coefficients) demonstrated a moderate correlation between IFA and ELISA and between CFT and IFA. On the other hand, statistically insignificant correlations were noted between CFT and ELISA. Statistically significant differences in percentage of positive results were recorded between the 3 different serological methods. However, no statistical significance between CFT and ELISA was recorded. IFA seems to be a good tool of serological diagnosis of Q fever in humans; however, laboratories use CFT and ELISA as well. Generally, previous studies have reported that CFT showed lower sensitivity compared to IFA, and ELISA is more intensive and time-consuming than IFA. On the basis of the literature data and the conducted studies, it should be noted that IFA is an important tool in the follow-up of patients and in identifying patients at risk for developing chronic Q fever [8]. The presented study confirms that the highest positive results for chronic form of Q fever in foresters were obtained by the IFA.

CONCLUSION

The presented study demonstrates that infections with *C. burnetii* are present in foresters in south-eastern Poland. Detection of antibodies against I and II phase of *C. burnetii* antigens in humans indicated that the chronic and acute forms of Q fever are presented in humans occupationally exposed to contact with ticks and wild animals. Prevalence of

C. burnetii infected ticks may indicate that these arthropods contribute to the transmission of Q fever in this endemic region. Due to the high risk of endocarditis in the chronic course of infection, seropositive humans should be included in specialist cardiological examinations. Comparison of 3 serological methods used showed that their diagnostic potential is differentiated, but that the most useful serological method is the IFA.

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