American Academic Scientific Research Journal for Engineering, Technology, and Sciences
ISSN (Print) 2313-4410, ISSN (Online) 2313-4402
http://asrjetsjournal.org/

Serological and Molecular Detection of *Coxiella Burnetii* in Clinical Samples from Veterinarians and Cattle Farm Workers from Gabrovo Region, Bulgaria

Petia Genova-Kalou[a]*, Stefka Krumova[b], Miroslav Parvanov[c], Radostina Stefanova[d], Radoslav Marinov[e], Ivona Andonova[f], George Dyankov[g], Konstantin Simeonov[h]

[a,b,d,e,f]*National Centre of Infectious and Parasitic Diseases (NCIPD), Virology Department, 44A "Gen. Stoletov" Blvd., 1233 Sofia, Bulgaria*

[c,g]*Bulgarian Veterinary Association, 15A Pencho Slaveykov Blvd., 1606 Sofia, Bulgaria*

[h]*National Diagnostic and Research Veterinary Medical Institute "Prof. Dr. G. Pavlov", Bulgarian Food Safety Agency (BFSA), 15A Pencho Slaveykov Blvd., 1606 Sofia, Bulgaria*

[a]*Email: petia.d.genova@abv.bg,* [b]*Email:stefka.krumova@gmail.com,* [c]*Email: miro_purvanov@abv.bg,* [d]*Email: rss_94@abv.bg,* [e]*Email: r.m.r@mail.bg,* [f]*Email: ivona_a@yahoo.com,* [g]*Email: ge.dyankov@gmail.com,* [h]*Email: kbsimeonov@yahoo.com*

Abstract

Coxiella burnetii, which causes Q fever, is a highly infectious agent that is widespread around the world. During the last decades, the number of cases reported in Bulgaria varied from year to year. The present study aimed to determine the frequency of *C. burnetii* infection using ELISA and conventional PCR among freelance veterinarians and cattle farm workers in Gabrovo province, Bulgaria. In the period April 2020 to June, 2021 a total of 154 blood samples of target group was tested in the National Reference Laboratory of Cell cultures, rickettsia and oncogenic viruses (NRL CCROV) at NCIPD - Sofia. Diagnosis of C. burnetii was performed by indirect enzyme-linked immunosorbent assay ELISA (anti-Coxiella burnetii ph. II IgG/IgM) and by end-point PCR technique (to detect the sodB gene region of C. burnetii). By indirect ELISA assay of the tested 154 clinical samples, anti-C. burnetii positive ph. II IgM antibodies were registered in 37% of samples. A relatively high percentage are affected in the active age between 50-60 years old. Anti-C. burnetii positive ph. II IgG antibodies were proven at 50% of tested samples.

---

* Corresponding author.

A positive PCR signal for C. burnetii DNA was obtained at 37/154 (20% of samples) and follows the above reported trend of acute infection of active age patients. Around 10% of tested samples were positive for three C. burnetii laboratory markers. We conclude that Q fever is endemic in Bulgaria. More research is necessary in different Bulgarian regions to set the human risk groups, to diagnose acute and chronic Q fever and to determine the economic impact of Q fever in the cattle industry. In the NRL CCROV was developed diagnostic scheme including complex methods to improve early laboratory diagnosis of C. burnetii, allowing taking proper treatment of suspected with Q fever patients.

Keywords: *Coxiella burnetii;* seroepidemiology; end-point PCR; freelance veterinarians and cattle farm workers; Gabrovo regions; Bulgaria.

1. Introduction

Zoonoses are constantly growing group of infections, which have emerged and reemerged as the foremost global security challenge throughout the world. They increasingly spread among human population like epizootic outbreaks and epidemics, with serious social, environmental and economic impact [1, 2]. Rapidly advancing climate changes, caused by global warming, increased trade in live animals and globalizing world, enhance existing natural and anthropurgic foci of several diseases with zoonotic potential [3, 4]. One of the top global priority zoonosis is Q fever, caused by intracellular gram-negative bacterium Coxiella burnetii, established on all continents, with the exception of New Zealand [5]. However, this infectious is still neglected and under-reported in most of countries, because the clinical signs in both acute and chronic forms of Q fever are nonspecific, the incubation period is relatively long, the levels of C. burnetii in clinical samples is too low, absent specific laboratory conformation and appropriate treatment protocols [6, 7]. Coxiella burnetii is included in category "B" list of potential biological weapons [8]. The host range susceptible to Q infection is too large and includes a variety of wild and domestic animals, birds and amphibians, and the ability of ticks to stored and transmitted as vectors Q infectious agent are associated with the formation of natural outbreaks [9, 10]. Infected animals, even if asymptomatic, can spread this bacterium to the environment through body fluids such as milk, urine, stool, cervical mucus, and fluids at the time of delivery or during miscarriage [11]. Transmission to humans is most frequently due to inhalation of aerosolized bacteria that are spread in the environment by infected animals and rarely alimentary [12]. Additionally, factors such as a large number of infected animals, farms located close to populated areas, and lack of epidemiological surveillance increase the incidence of infection [12]. Clinical manifestation of acute Q fever in humans can be asymptomatic (50%), but there can also be signs including atypical pneumonia, granulomatous hepatitis, meningioencephalitis and endocarditis [13, 14]. Infectious endocarditis is the most common form of chronic Q fever infection and patients with valvular and vascular lesions are at high-risk and is fatal if untreated [15, 16]. Because the clinical presentation is similar to that of other diseases, Q fever often remains underdiagnosed [12, 13]. Laboratory diagnosis of acute Q fever is ideally based on a combination of PCR and serology in blood and serum samples [16, 17]. IgM Phase II is still detectable 12 months after transmission of *C. burnetii* in 62-83% of the serum samples [18]. Seroconversion or a 4-fold increase in the IgG phase II titer is used to confirm the diagnosis of acute Q fever [19]. PCR has been shown to be positive for almost all early acute Q fever patients that have not yet an antibody response and in almost all of those that have peak an IgM phase II antibody response [20]. Concerning Q fever in humans,

current epidemiological studies indicate that this zoonosis should be considered a public health problem in many countries, including Netherlands, France, the United Kingdom, Italy, Spain, Germany, Israel and Greece, as well as in many countries where Q fever is prevalent but unrecognized because of poor surveillance of the disease [21 – 27]. In Bulgaria, Q fever in humans was first recognized by Mitov and his colleagues in 1949 [28]. For more than 60 years, numerous sporadic cases and small and large epidemics, involving tens to hundreds of persons, occurred in different regions of the country [29, 30]. Based on previous studies 2017, in Gabrovo region, the presence of *C. burnetii* has been confirmed among the livestock and humans from several villages of the area [31]. The present study aimed to determine the frequency of *C. burnetii* infection using ELISA and conventional PCR among freelance veterinarians and cattle farm workers in Gabrovo province, Bulgaria.

2. Materials and Methods

In this study, we assessed the seroprevalence rate of *Coxiella burnetii* and to confirm with molecular technique in blood samples of veterinarians, who practice hourly in various livestock farms in Gabrovo region, as well as and cattle farm workers of the region from April, 2020 till June, 2021.

2.1. Area of study and population

Gabrovo province is a small province lying at the geographical centre of Bulgaria on the territory 2026,005 km^2. Gabrovo district is divided territorially and administratively into 4 municipalities, bearing the names of their main cities and included 344 villages. In 2009 the total population of the area is 130,001. The leading areas of animal husbandry in the province are cattle and sheep breeding (Fig. 1).

Figure 1: Gabrovo province is located in the geographical centre of Bulgaria in the north part of Stara Planina

2.2. Patient characteristics

The study was conducted at the National Centre of Infectious and Parasitic Diseases – Sofia (Bulgaria) in the National Reference Laboratory of Cell cultures, rickettsia and oncogenic viruses (NRL CCROV). The study group was 154 patients (103 men and 51 women). Details of their clinical history were recorded on admission. Cases and positive samples were described by region, age group, sex and laboratory test. All the patients gave their informed consent before being included in the study. In generally, study patients were of Bulgarian nationality and lived in Gabrovo province.

2.3. Coxiella burnetii serological studies

Blood samples were taken on admission by venipuncture from each patient and tested for anti-*C. burnetii* IgM and IgG phase II antibodies by indirect enzyme-linked immunosorbent assay ELISA (Euroimmun, Lűbeck, Germany). Blood was centrifuged at 4000*g* for 10 min, serum was aliquoted and stored for a maximum of 3 days at 4°C before use in the Q fever-ELISA and then harvested and frozen at -80^{o}C until further analysis. According to data provided by the manufacturer anti-*C. burnetii* IgM/IgG phase II ELISA tests have a sensitivity respectively of 91.7% / 97.1% and a specificity of 90% / 91.8%. The ELISA tests were performed according to the manufacturer's instructions. Both tests were a semi-quantitative, in which results are expressed as a ratio of extinction value of the control or patient sample over extinction value of the calibrator (ratio = extinction of the control or patient sample/extinction of the calibrator). In Euroimmun test ratio ≥ 1.1 was taken as positive. Samples were negative if the absorbance value was < 0.8.

2.4. Molecular detection of Coxiella burnetii

Whole blood samples were taken on admission by venipuncture from each patient and collected into in ethylenediaminetetraacetic acid (EDTA Becton, Dickinson and Company, USA) blood tubes. The samples were handled under sterile conditions to avoid cross-contamination. Following collection, samples were transported on ice to the NRL CCROV, where whole blood samples were centrifuged, aliquots for DNA extraction, and rest plasma stored at -80°C for further analysis. DNA from blood samples were extracted with the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The genomic DNA of each sample was stored at -20°C under sterile conditions. The extracted DNA was subjected to an end-point PCR assay. To the reaction mixture, prepared at a volume of 22.5 µl in 0.2-ml test tubes for each sample, was added 2.5 µl of the DNA extract, and amplification was performed as described by Stein A, Raoult D. (1992) [32]. For the *sodB* gene, using the specific primers CB1 and CB2, which product is 257 bp in length and is specific to the DNA of *C. burnetii* CB1 (5' ACT CAA CGC ACT GGA ACG GC 3') and CB2 (5' TAG CTG AAG CCA ATT CGC C 3') primers were used. Following amplification, electrophoresis was performed in 1.5%agarose gels, and the results were imaged

2.5. Statistical analysis

The tests included frequency calculation, frequency percentage, and chi-square test. Variables with control distribution are presented as mean and standard deviation (SD).

3. Results and Discussion

In the period April, 2020 till June, 2021 154 study patients, 67% were man and 33% women. The tested patients are from endemic for Q fever Bulgarian region (Gabrovo) in which were report small epidemic outbreak in 2017 [31]. Age range of the participants was 18 - 70 years. By indirect ELISA assay of the tested clinical samples, anti-*C. burnetii* positive ph. II IgM antibodies were registered at 57/154 (37% of samples). A relatively high percentage are affected in the active age between 50-60 years old, with a predominance of men (about 70%). Anti-*C. burnetii* positive ph. II IgG antibodies were proven at 77/154 (50%). A positive PCR signal for *C. burnetii* DNA was obtained at 37/154 (20%) and follows the above reported trend of acute infection of active age patients. Around 10% of tested samples were positive for three *C. burnetii* laboratory markers (Figure 2). The presence of anti-*Coxiella* IgG ph. II antibodies was positively associated with increasing age: very few reactive antibodies were found in youths under 20 years of age, while they positivity increased from 15% in 21–30 year olds to 29% in adults over 30 years of age (Fig. 2). Patients in the age group 50-60 years have a typical acute Q fever clinical manifestation (fever of unknown origin, headache, myalgia) with the highest frequency. PCR has been shown to be positive for almost all early acute Q fever patients that have not yet an antibody response and this method can be used as a first choice for diagnosis during an epidemic outbreak. A history of contact with animal birth products was significantly related to *C. burnetii* seropositivity.

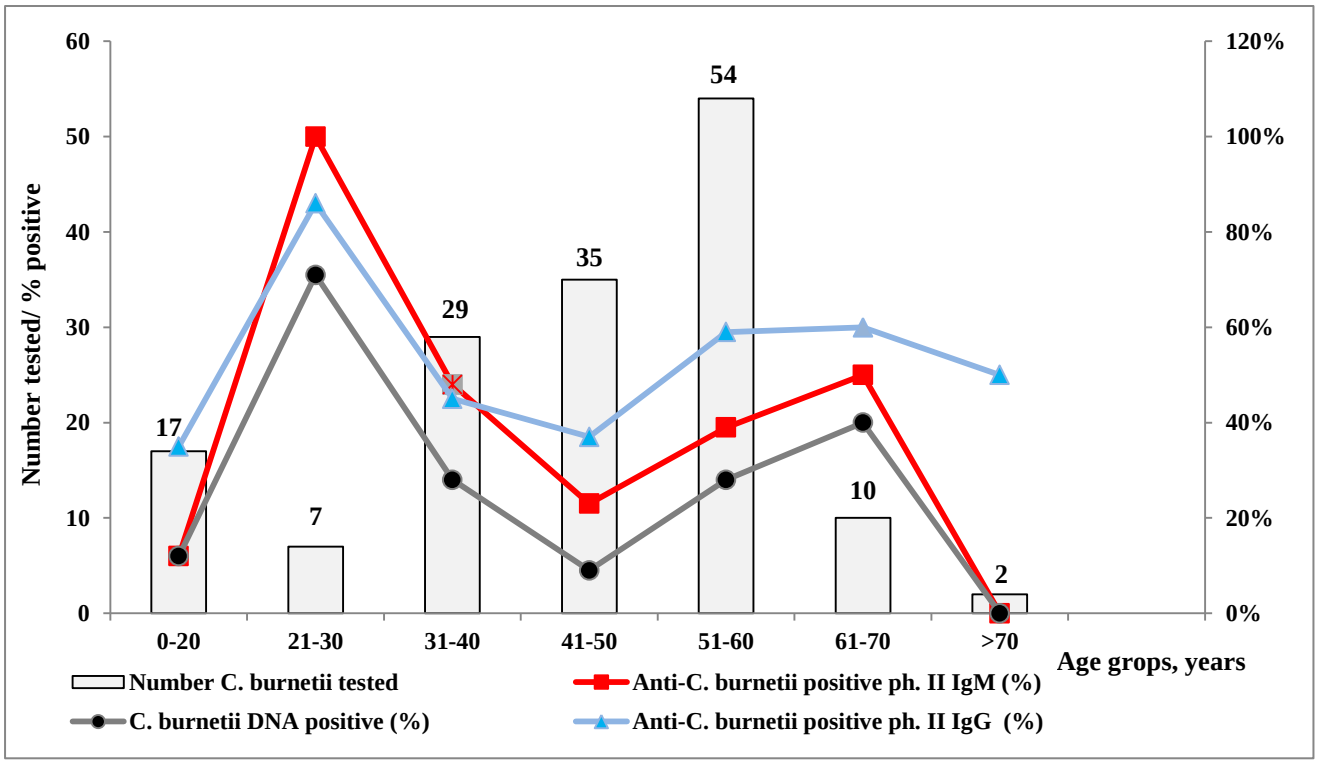

Figure 2: Distribution of proven positive patient samples for *C. burnetii* with a combination of diagnostic markers in percentages by age groups (n = 154)

4. Conclusions

We conclude that Q fever is endemic in Bulgaria but that reporting only has been done in research studies. More

research is necessary in different areas of Bulgaria to set the human risk groups, to diagnose acute and chronic Q fever and to determine the economic impact of Q fever in the cattle industry. Concerning acute Q fever, we propose the physicians in Bulgaria to request testing for acute Q fever in cases of atypical pneumonia in risk groups like cattle farm workers, veterinaries and slaughterhouse workers. Although *C. burnetii* occurs in most parts of the world, the prevalence of infections in humans is often underestimated because definitive testing is not carried out and signs are generally subclinical or confused with more common diseases [33, 34]. Diagnosis is of key importance to the control, monitoring and adequate antibiotic treatment of Q fever. The results of this study indicated that indirect ELISA assay and PCR were a sensitive method for proving of acute *C. burnetii* infection. Our findings also call for further investigation into the clinical relevance of chronic Q fever in different regions in Bulgaria. Knowledge about chronic Q fever is limited, but the studies that have been conducted showed that about 5% of the patients who got infected with *C. burnetii* would develop chronic Q fever. Additional serological and clinical monitoring is recommended for the patients detected in this study to determine if they have high anti-*C. burnetii* IgG phase I antibody titers. In the NRL CCROV was developed diagnostic scheme including complex methods to improve early laboratory diagnosis of *C. burnetii*, allowing taking proper treatment of suspected with Q fever patients.

Acknowledgements

This study was supported by the Bulgarian National Science Fund under Grant No KP-06-N33/3/2019. Title: "Molecular genetic identification and creation of an archival genomic bank of the circulating human and animal *C. burnetii* genotypes and determination of their role as particular dangerous infectious agents causing epizootic and epidemiological outbreaks on the territory of the Republic of Bulgaria".

Reference

[1]. Jones K, Patel N, Levy M, et al. (2008). “Global trends in emerging infectious diseases”. Nature. 451:990-94. Available: https://doi.org/10.1038/nature06536

[2]. Karesh W, Dobson A, Lloyd-Smith J, et al. (2012). “Ecology of zoonoses: natural and unnatural histories”. Lancet. 380:1936–45. Available: https://doi.org/10.1016/S0140-6736(12)61678-X.

[3]. Mishra J., Mishra P. & Arora N. (2021). “Linkages between environmental issues and zoonotic diseases: with reference to COVID-19 pandemic”. Environmental Sustainability. Available: https://doi.org/10.1007/s42398-021-00165-x

[4]. Campbell-Lendrum D, Manga L, Bagayoko M, Sommerfeld J. (2015). “Climate change and vector-borne diseases: what are the implications for public health research and policy?”. Philos Trans R Soc Lond B Biol Sci. 370(1665):20130552. Available: https://doi.org/10.1098/rstb.2013.0552

[5]. Brook C, Dobson A. (2015). “Bats as ‘special’ reservoirs for emerging zoonotic pathogens”. Trends in Microbiology. 23: 172– 180. Available: https://doi.org/10.1016/j.tim.2014.12.004

[6]. Porter S, Czaplicki G, Mainil J, Guattéo R, Saegerman C. (2011). “Q Fever: current state of knowledge and perspectives of research of a neglected zoonosis”. Int J Microbiol. 248418. Available: https://doi.org/10.1155/2011/248418

[7]. Eldin C, Mélenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, et al. (2017) “From Q Fever to

Coxiella burnetii Infection: a Paradigm Change". Clin Microbiol Rev. 30(1):115–90. pmid:27856520. Available: https://doi.org/10.1128/CMR.00045-16

[8]. Kagawa F, Wehner J., Mohindra. (2003) "Q fever as a biological weapon". Eur PMC, 18, 183 - 195.

[9]. Woldehiwet Z. (2004). "Q fever (coxiellosis): epidemiology and pathogenesis". Res Vet Sci. 77:93–100. pmid:15196898. Available: https://doi.org/10.1016/j.rvsc.2003.09.001

[10]. Tissot-Dupont H, Raoult D. (2008, Sep) Q fever. Infect Dis Clin North Am. 22(3):505-14, ix. Available: https://doi.org/10.1016/j.idc.2008.03.002

[11]. Angelakis E, Raoult D. (2010). "Q fever". Vet Microbiol. 140: 297–309. Available: https://doi.org/10.1016/j.vetmic.2009.07.016

[12]. Arricau-Bouvery N, Rodolakis A. (2005). "Is Q Fever an emerging or re-emerging zoonosis?". Veterinary Research, BioMed Central. 36 (3), pp.327-349. Available: https://hal.archives-ouvertes.fr/hal-00902979

[13]. Honarmand H. (2012). "Q Fever: An Old but Still a Poorly Understood Disease", Interdisciplinary Perspectives on Infectious Diseases, vol. 2012, Article ID 131932, 8 pages. Available: https://doi.org/10.1155/2012/131932

[14]. Healy B, Llewelyn M, Westmoreland D, Lloyd G, Brown N. (2006). "The value of follow-up after acute Q fever infection," Journal of Infection, vol. 52, no. 4, pp. e109–e112.

[15]. Million M, Walter G, Thuny F, Habib G, Raoult D. (2013, Sep). "Evolution from acute Q fever to endocarditis is associated with underlying valvulopathy and age and can be prevented by prolonged antibiotic treatment". Clin Infect Dis. 57(6):836-44. Available: https://doi.org/10.1093/cid/cit419

[16]. Wegdam-Blans MC, Vainas T, van Sambeek MR, et al. (2011). "Vascular complications of Q-fever infections". Eur J Vasc Endovasc Surg. 42(3):384–392. Available: https://doi.org/10.1016/j.ejvs.2011.04.013

[17]. Fournier P, Raoult D. (2003). "Comparison of PCR and serology assays for early diagnosis of acute Q fever|. J. Clin. Microbiol. 41:5094–5098. Available: https://doi.org/10.1128/JCM.41.11.5094-5098.2003

[18]. Schneeberger P, et al. (2010). "Real-time PCR with serum samples is indispensable for early diagnosis of acute Q fever". Clin. Vaccine Immunol. 17:286–290. Available: https://doi.org/10.1128/CVI.00454-09

[19]. Wegdam-Blans M, Wielders C, Meekelenkamp J, et al. (2012). "Evaluation of commonly used serological tests for the detection of Coxiella burnetii antibodies in well-defined acute and followup sera". Clin Vaccine Immunol. 19:1110–1115.

[20]. Dupuis G, Péter O, Peacock M, Burgdorfer W, Haller E. (1985). "Immunoglobulin responses in acute Q fever". J. Clin. Microbiol. 22:484–487. Available: https://doi.org/10.1128/jcm.22.4.484-487.1985

[21]. Aitken ID, Bögel K, Cračea E, et al. (1987). "Q fever in Europe: current aspects of aetiology, epidemiology, human infection, diagnosis and therapy". Infection. 15(5):323–327. Available: https://doi:10.1007/BF01647731

[22]. Frankel D, Richet H, Renvoisé A, Raoult D. (2011). "Q fever in France, 1985-2009". Emerg Infect Dis. 17(3):350-356. Available: https://doi.org/10.3201/eid1703.100882

[23]. Amitai Z, Bromberg M, Bernstein M, Raveh D, Keysary A, David D, et al. (2010). "A large Q fever

outbreak in an urban school in central Israel". Clin Infect Dis. 50:1433–8. Available: https://doi.org/10.1086/652442

[24]. Enserink M. (2010). "Infectious diseases. Questions abound in Q-fever explosion in the Netherlands". Science. 327:266–7. Available: https://doi.org/10.1126/science.327.5963.266-a

[25]. Hellenbrand W, Breuer T, Petersen L. (2001). "Changing epidemiology of Q fever in Germany, 1947–1999". Emerg Infect Dis. 7:789–96. Available: https://doi.org/10.3201/eid0705.010504

[26]. Wallensten A, Moore P, Webster H, Johnson C, van der Burgt G, Pritchard G, et al. (2010). "Q fever outbreak in Cheltenham, United Kingdom, in 2007 and the use of dispersion modelling to investigate the possibility of airborne spread". Euro Surveill. 15:19521. Available: https://doi.org/10.2807/ese.15.12.19521-en

[27]. Anderson A. et al. (2013). "Diagnosis and management of Q fever–United States, 2013: Recommendations from CDC and the Q Fever Working Group". MMWR Recomm. Rep. 62, 1–30. Available: https://www.cdc.gov/mmwr/preview/mmwrhtml/rr6203a1.htm

[28]. Mitov A, Shindarov L, Serbezov V. (1959). "Q fever in Bulgaria". Mod Med. 1-2:39-46.

[29]. Serbezov V, Kazár J, Novkirishki V, Gatcheva N, Kovácová E, Voynova V. (1999). "Q fever in Bulgaria and Slovakia". Emerg Infect Dis. 5(3):388-94. Available: https://doi.org/10.3201/eid0503.990309

[30]. Kamenov G, Tiholova M. (2004). "Q fever outbreak in Botevgrad, Bulgaria: May-June 2004". Euro Surveill. 8(35):2535. Available: https://doi.org/10.2807/esw.08.35.02535-en

[31]. Genova-Kalou P, Vladimirova N, Stoitsova S, Krumova S, Kurchatova A, Kantardjiev T. (2019). "Q fever in Bulgaria: Laboratory and epidemiological findings on human cases and outbreaks, 2011 to 2017". Euro Surveill. 24(37):1900119. Available: https://doi.org/10.2807/1560-7917.ES.2019.24.37.1900119

[32]. Stein A, Raoult D. (1992). "Detection of Coxiella burnetti by DNA amplification using polymerase chain reaction". J Clin Microbiol. 30(9):2462-6. Available: https://doi.org/10.1128/jcm.30.9.2462-2466.1992

[33]. Mathews K, Toribio J, Norris J, Phalen D, Wood N, Graves S, Sheehy P, Bosward K. (2020, Nov). Coxiella burnetii seroprevalence and Q fever in Australian wildlife rehabilitators. One Health; 12:100197. Available: https://doi: 10.1016/j.onehlt.2020.100197.

[34]. Miller H, Kersh G. (2020, Dec). Analysis of recombinant proteins for Q fever diagnostics. Sci Rep. 1; 10(1):20934. Available: https://doi: 10.1038/s41598-020-77343-0.