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Article

# First Insight into the Incidence of *Coxiella burnetii* Infection among Veterinary Medicine Students from Bulgaria

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**Abstract:** This study is aimed to assess the prevalence of *Coxiella burnetii* infection among veterinary medicine students from two Bulgarian Universities, located in Sofia and Stara Zagora. A total of 185 veterinary students blood samples were collected and tested for the detection of *C. burnetii* phase II antibodies and presence of DNA using an enzyme-linked immunosorbent assay (ELISA) and end-point PCR test, respectively. Out of all samples, 29.7% were positive for at least one *C. burnetii* phase II antibody marker or by PCR test. Veterinary students from Stara Zagora showed significantly seropositive status of Q fever (33.6%), then those who studied in Sofia (23%). The evidence for recent exposition with detection of anti-*C. burnetii* IgM (+) antibodies had 14.6%. Seroprevalence among students in Stara Zagora was higher (15.3%). Anti-*C. burnetii* phase II IgG antibodies were detected in 21.6% examined samples. Our study revealed a higher seropositivity among male students (32.8%) as compared to the female ones (16.0%). End-point PCR assay detected as positive 5.9% blood samples. The relative risk (RR) of Q fever exposure for male students was 40.7%, whereas for female (24.6%). Findings from this study indicated that the *C. burnetii* infection is widely distributed amongst veterinary students from Bulgaria. The study emphasizes the need for improved safety protocols and infection control measures in veterinary training programs.

**Keywords:** Bulgaria; *Coxiella burnetii*; Q fever; students; veterinary medicine

## 1. Introduction

Q fever is endemic zoonosis with global distribution, caused by intracellular bacterium *Coxiella burnetii* and with great human and animal health significance [1–3]. The last few years, the highest numbers of confirmed cases of Q fever in Europe were reported in Spain, France, Germany, Romania, Hungary and Bulgaria [4–9]. The primary source of infection to humans are domestic ruminants, such as goats, cattle, sheep, as well as pets, in which the infection is usually asymptomatic, but it could be also associated with infertility, abortions and premature birth [10–13]. The clinical manifestations of Q fever in human varies from non-specific, self-limiting disease to an acute form (fever of unknown origin, atypical pneumonia, granulomatous hepatitis) [14–16] or chronic presentation (endocarditis, encephalopathy, etc.) [17,18]. Laboratory diagnosis of acute cases is based on: (i) isolation and cultivation, which requires BSL3 facilities for tissue cultures [19]; (ii) serological tests, such as indirect immunofluorescence (IFA), recognized as the gold standard and enzyme-linked immunosorbent assay (ELISA) [20,21]; (iii) antigen detection assay such as immunohistochemical staining (IHC) [22]; and (iv) molecular detection of *C. burnetii* DNA by different PCR techniques for early diagnosis of acute phase [23,24]. The anti-*C. burnetii* antibodies response is detectable after 2-3 weeks following infection. The level of anti-*C. burnetii* IgM phase II increases rapidly after infection and are detectable for several months as a marker of recent infection. The presence of specific IgG phase II provides evidence of a recent *C. burnetii* infection or a past exposure and can remain detectable for years [25,26].

Due to the fact that *C. burnetii* is environmentally stable and can be transmitted from animal hosts to humans mainly via inhalation of contaminated aerosols or dust particles [27,28], the most vulnerable group are people who are in the higher occupational risk of exposure and daily contact with farm animals and pets [29–31]. Veterinary medicine students remain at high risk of being infected with *C. burnetii* during sixth-year veterinary curriculum, since this period from their training cover animal handling and treatment a wide variety of species [32]. According to the data of scientific literature, few serological studies have reported on the seroprevalence for *C. burnetii* among veterinary medicine students, [33–35]. Only few studies have assessed zoonotic risks for veterinary medicine students [36–38].

Q fever is considered endemic in Bulgaria, however, due to the nonspecific and pleomorphic clinical presentation of the *C. burnetii* infection it often remains neglected and underdiagnosed. Thus, little is known about seroprevalence of Q fever among various professional groups, occupationally exposed to infection with *C. burnetii*, including veterinary medicine students, because of their close contact with infected livestock and pets.

Therefore, the aim of the present study was to assess the prevalence of *C. burnetii* infection among veterinary medicine students at two Veterinary Medicine Faculties (Sofia and Stara Zagora), located in western and central part of the country. To the best of our knowledge, this is the first evidence of *C. burnetii* infection in veterinary medicine students from Bulgaria.

## 2. Materials and Methods

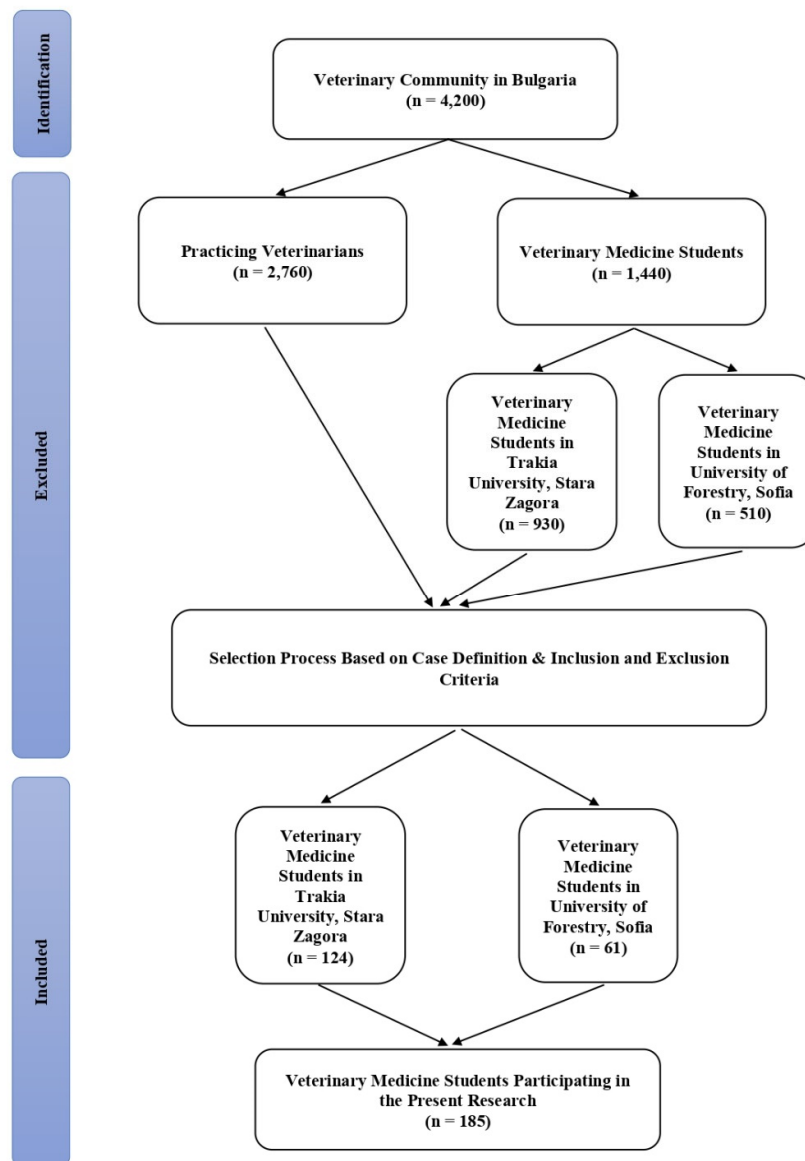
### 2.1. Target Population and Sample Collection

In the present study, blood samples were taken from a total of 185 veterinary medicine students, in the 5th year of study during the academic year 2022–2023 from the two Veterinary Faculties at the University of Forestry in Sofia and Trakia University in Stara Zagora, Bulgaria, respectively. Participants in the current research were selected based on case definition as well as the inclusion and exclusion criteria (Figure 1).

The students, included in the study have to meet the following criteria: (a) age > 18 years; (b) persons, studying in a specialty “Veterinary Medicine” in the last but one year of study; (c) participants, in the end of their curriculum, which are in close contact with farm animal and pets during practical training; (d) obtained written informed consent from each student to participate in a scientific survey; (e) female participants, who did not report a possible pregnancy at the moment of the survey. The exclusion criteria for the current research include: (a) individuals younger than 18

years; (b) persons from other university specialties (students of specialties, such as mathematics, engineering, finance and economics, law, human sciences, etc); (c) individuals with contraindications for venepuncture; (d) persons who refused to give written informed consent; (e) participant with Q fever vaccination history; (f) persons with a severe clinical form of an acute or chronic disease.

The samples from students were taken during routine diagnostic tests following standard procedure. After obtaining a written consent and completing a questionnaire, students were invited to donate a blood sample of 10 ml by venepuncture for testing. Blood samples were delivered to the National Reference Laboratory (NRL) "Rickettsia and cell cultures", Department of Virology, National Centre of Infectious and Parasitic Diseases, Sofia (Bulgaria). Sera were separated by centrifugation at 1500 rpm for 10 min, transferred into sterile vacuum tubes and stored at  $-20^{\circ}\text{C}$  until processed. Whole blood samples ( $200\mu\text{L}$ ), collected in EDTA tubes were used for DNA extraction.



**Figure 1.** Flow chart showing the selection process of research participants.

## 2.2. Case Definition

The presence of anti-*C. burnetii* phase II IgG antibody positivity was assumed to indicate a possibly acute or a history of past *C. burnetii* infection, i.e. previous encounter with this pathogen. Acute phase of *C. burnetii* infection was defined when one of the following criteria were present of: (a) a 4-fold increase of the IgG phase II antibodies titer to *C. burnetii* between paired serum samples as evidence by ELISA; (b) positive ELISA result for IgM antibodies against phase II antigens of *C. burnetii*; (c) detection of the presence of *C. burnetii* DNA in EDTA blood specimens via amplification of a specific target by PCR assay, < 21 days of onset of disease; (d) a negative blood PCR result but a positive ELISA result for IgM and IgG against phase II antigens of *C. burnetii*.

## 2.3. Serological Tests

The serum samples were examined for the presence of anti-*C. burnetii* phase II IgM and IgG antibodies using a commercial ELISA kit (EUROIMMUN, Lübeck, Germany) according to the manufacture's recommendations. The absorbance values were determined at 450 nm (reference wavelength of 620–690 nm) using an Epoch ELISA reader (BioTek Instruments, Inc., VT, USA), on the Gen5™ data analysis software (BioTek Instruments, Inc., VT, USA). Optical density (OD), cut-off values and controls were checked. Results were evaluated semiquantitatively by calculating a ratio (R) of the extinction value of the patient sample over the extinction value of the calibrator. The results were categorised as follow:  $R \geq 1.1$  was taken positive and indicating a possibly acute or evidence for past infection;  $R < 0.8$  considered as negative and  $R \geq 0.8$  to  $< 1.1$  indicated as suspicious (equivocal) [39].

## 2.4. PCR Assay

Whole blood samples were tested for the presence/absence of *C. burnetii* DNA using end-point PCR by specific primers. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. DNA was eluted with forty microliters of elution buffer and stored at 4°C until use. The primers were designed to amplify a 687-bp fragment of the transposon-like repetitive region of the bacterial genome (*IS1111*) of *C. burnetii*, using Trans-1 and Trans-2 primers with the following sequence: Trans-1 (5'-TAT GTA TCC ACC GTA GCCAGT C-3') and Tans-2 (5'-CCC AAC AACACC TCC TTATTC-3') (Metabion, Germany) [40]. The Trans-PCR was carried out as described [41]. The resulting amplicons were electrophoresed on 1.5% agarose gel and visualized under UV light [42].

## 2.5. Statistical Analysis

Data analysis was performed with the help of SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA) and Excel 2016 (Microsoft, Redmond, WA, USA). Data were entered and arranged in MS Excel. The risk exposure of *C. burnetii* among different veterinary student groups, categorized by sex and location, was calculated. Statistical analysis was performed to compare the relative risks (RR) as proportion of exposed over unexposed students. A Chi-squared test was utilized for this comparison, and a P-value < 0.05 was considered statistically significant. The final results obtained from the statistical analysis were confirmed by all scientists involved in this study.

## 2.6. Ethical Considerations

All veterinary medicine students signed a written informed consent to participate in scientific research. Participants gave written informed consent for the invasive procedures conducted on them (venepuncture). Every student had access to their laboratory result. Scientists participating in this research conducted their activities according to the ethical principles of Declaration of Helsinki (adopted in June 1964, last revision in October 2013). The current survey was approved by the Local Ethics Committee of National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria (NCIPD-02/07 February 2023), who confirmed that the research was in full accordance with all ethical



principles and practices. The authors have not used artificial intelligence (AI) to create this manuscript.

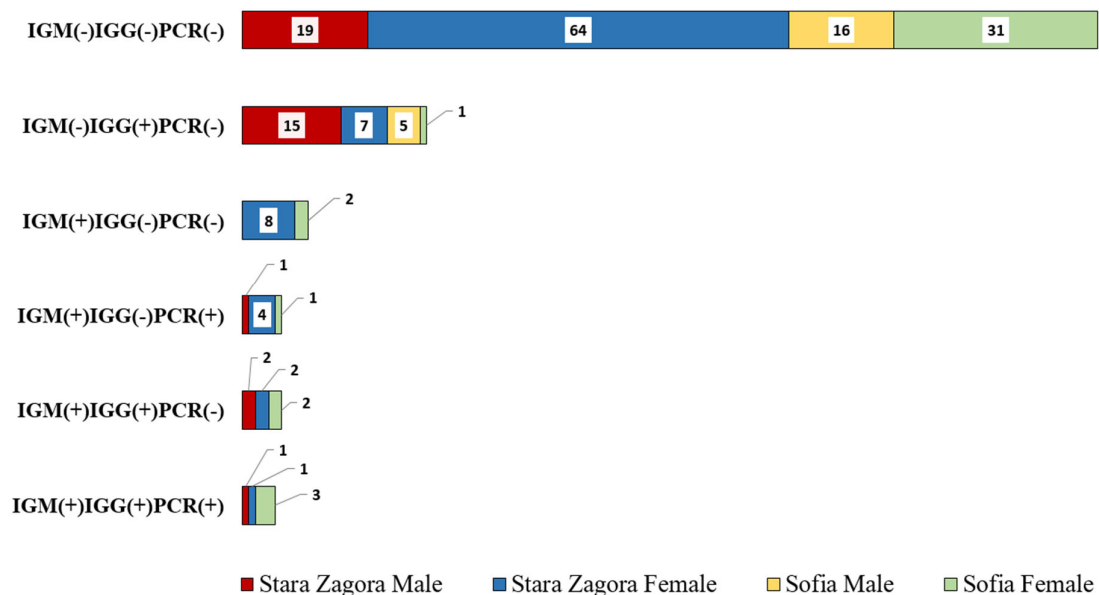
### 3. Results

#### 3.1. Characterization of Study Population

A two-step approach was used to discriminate acute from past *C. burnetii* infection. At first, all serum samples were screened by a commercial ELISA kit for the presence of anti-*C. burnetii* phase II IgM and IgG antibodies. Then the positive, were analysed by end-point PCR assay, targeting *IS1111* transposable element, which was routinely used marker in the epidemiological investigation of *C. burnetii* infections.

#### 3.2. Serology Analysis

The result in Figure 2 showed the distribution of persons with various immune responses and PCR results across four groups: Stara Zagora Male, Stara Zagora Female, Sofia Male, and Sofia Female. The categories include combinations of IgM, IgG, and PCR results, indicating different stages and types of exposure or infection with *C. burnetii*. Out of all 185 serum samples studied, 55 (29.7%) were positive for at least one marker anti-*C. burnetii* antibody for phase II or presence of *C. burnetii* DNA (Chi-squared (1) = 27.88;  $P < 0.001$ ). Veterinary students from Stara Zagora showed significantly more frequent seropositive status of Q fever, than those who studied in capital city of Sofia, (42 out of 125 (33.6%) vs 14 out of 61 (23%), Chi-squared (1) = 2.22;  $P < 0.05$ ). By sex, 24 out of 59 (40.7%) of male samples were positive, whereas 31 out of 126 (24.6%) of female samples were positive (Chi square (1) = 4.00,  $P < 0.05$ ).



**Figure 2.** Distribution of exposed and unexposed to *C. burnetii* veterinary students, assessed by immune status or pathogen DNA identification by PCR in the venous blood by sex and location.

Among all tested veterinary students, who provided a serum samples, 28 (14.6%) were seropositive for anti-*C. burnetii* phase II IgM antibody and therefore had evidence for recent exposition. Of those, 4 (14.8%) were male and 23 (85.2%) were female (Chi square (1) = 3.98,  $P < 0.05$ ). Eight of 61 serum samples (13.1%) from veterinary students in Sofia were positive to phase II IgM antibodies. Seroprevalence among students in Stara Zagora was 15.3% (19/126) (Chi square (1) = 0.14,  $P = 0.71$ ).

Presence of anti-*C. burnetii* IgG antibody phase II was detected in 40 out of 185 (21.6%) examined serum sample. The seroprevalence for phase II antibodies to IgG reached in our group of subjects were 14.8% and 25%, respectively for University of Forestry and Trakia University, however this observation does not reach statistical significance (Chi square (1) = 2.44,  $P > 0.1$ ). It should be noted that the frequency of positive serum titer for Q fever (phase II) was greater in man than women, Chi square (1) = 6.84,  $P < 0.01$ . Moreover, incidences of seropositive for IgG male subjects were almost as twice as much in males (39%) then in females (13.5%) (Chi square (1) = 6.84,  $P < 0.01$ ). Of the 10 patients with suspected acute Q fever infection (presence of both class phase II anti-*C. burnetii* IgM/IgG(+) antibodies), the proportion of women was higher than that of man.

### 3.3. Molecular Analysis

In the current study, EDTA blood samples were further screened by end-point PCR assay for detection of *C. burnetii* DNA. End-point PCR assay detected as positive at 687bp by Trans-1 and Trans-2 primers 11 of 185 (5.9%) blood samples. In all, 7.1% of the females and 3.4% of the males tested positive.

### 3.4. Relative Risk Assessment Q Fever Exposure

The relative risk (RR) of Q fever exposure among veterinary students was assessed by comparing the prevalence of anti-*C. burnetii* antibodies between different groups, specifically focusing on sex and University affiliation (Table 1). For clarity all cases showed at least one positive test marker (IgG, IgM or PCR) were pooled together and designated as “exposed” and were compared to frequency of negative cases (“unexposed”). The relative risk of Q fever exposure for students in Stara Zagora was 33.1% and 23% in Sofia, respectively. The relative risk (RR) of Q fever exposure for male students was 40.7%, whereas for female students, the RR was 24.6%. Location-by-sex RR analysis showed that the relative risk of Q fever exposure for male students in Stara Zagora is 50%, followed by female students in Stara Zagora (25.6%). Male veterinary students in Sofia had an RR of 23.8%, and female students in Sofia had an RR of 22.5%.

**Table 1.** Association between *C. burnetii* exposition with sex and location variables. Relative risk for each experimental group is calculated.

Variables	Total, n	Exposed, n (%)	Unexposed, n (%)	Chi-squared statistics (df)	P-value	Relative Risk, %
<b>Pooled Sex</b>						
Males	59	24 (40.6)	35 (59.4)	2.22 (1)	< 0.05	41
Females	126	31 (24.6)	95 (75.4)			25
<b>Pooled Location</b>						
Sofia	61	14 (23.0)	47 (77.0)	4.00 (1)	< 0.05	23
Stara Zagora	124	41 (33.1)	83 (66.9)			33
<b>Males</b>						
Sofia	21	5 (23.8)	16 (76.2)	3.89 (1)	< 0.05	24
Stara Zagora	38	19 (50.0)	19 (50.0)			50

Females						
Sofia	40	9 (22.5)	31 (77.5)	0.14 (1)	> 0.1	23
Stara Zagora	86	22 (25.6)	64 (74.4)			26

#### 4. Discussion

Q fever is among the most common zoonotic diseases and even information for some countries is limited, available data showed a high prevalence of infection, particularly amongst the people, professionally involved in animal husbandry. The results of our study are in agreement with similar data reported by other authors from Europe. In 2000, Valencia et al. reported 11.02% *C. burnetii* seroprevalence in veterinary students from Zaragoza, Spain [38]. Turkish authors found 7.0% and 8.0% *C. burnetii* antibodies among 83 veterinarians from two distinct geographic regions [44]. German scientists detected 38.0% positive sera for *C. burnetii* phase II IgG antibodies among 424 veterinarians [45]. In 2012, de Rooij et al. reported on 18.7% IgG antibodies against *C. burnetii* in Dutch veterinary medicine students [36]. One year later, another Dutch scientific team found 65.1% antibodies against *C. burnetii* among 189 livestock veterinarians [46]. Similar data are reported also by other authors [47–49]. In short, our results and data from other authors from Europe show a moderate and high level of *C. burnetii* seropositivity among risk groups (persons in contact with animals; predominantly veterinarians).

To assess the importance of *C. burnetii* infection among risk groups (veterinarians, hunters, etc.), it is recommended to make a comparison with seropositivity in the general population. Tiscione et al. reported lower levels of *C. burnetii* antibodies among an urban group with no potential animal contact (6.1%) compared to the higher levels, found in a rural group with higher potential exposure to animals (49.1%) [50]. A few years later, Spanish authors found 3.0% positive serum samples for Q fever in healthy population from Lanzarote, Canary Islands [51]. Irish scientists detected 12.8% positive results for *C. burnetii* among 2,394 randomly selected subjects (humans) from the northern part of the country [52]. By testing plasma samples obtained from individuals living in Southern Netherlands, Brandwagt et al [53] reported significant dynamics in Q fever seroprevalence levels, ranging from 62.5%, as detected in 1983 to 14.4% in 1987, and decreasing to 1% in 2008 and 2.3% in 2010, respectively. Similar data are reported also by other authors [54–57]. All this clearly showed that there is a lower seropositivity for *C. burnetii* infection in the general population compared to the risk groups. This confirms the role of *C. burnetii* as an occupational disease pathogen and highlights the need for more detailed epidemiological investigations of infection amongst them, especially.

In our survey, high prevalence rates of antibodies against *C. burnetii* (30.8%) among veterinary medicine students, were found. The potential reasons for this fact can be many and of a different nature. As a first, Q fever is endemic to Bulgaria, and high environmental contamination can be expected due to the wide prevalence of *C. burnetii* infection found amongst ruminant herds/flocks in the country (Simeonov K, unpublished data). In addition, *C. burnetii* infection is easily acquired by inhaling of contaminated aerosol, which facilitates the transmission to people and the spread of the bacterium. The lack of protection skills in veterinary medicine students also increases the risk of infection when working with animals.

The elevated seroprevalence of anti-*C. burnetii* IgM (+) phase II antibodies among students in Trakia University suggests a direct correlation with their frequent contact with ruminants. The practical courses that involve hands-on livestock handling and farming activities contribute to increased exposure to *C. burnetii*. This cohort of students exhibited the highest seropositivity for anti-*C. burnetii* IgG (+) phase II antibodies. The curriculum at Trakia University includes a number of practical lessons involving farm animals, which lead to increased exposure to *C. burnetii* and a higher risk of infection. In contrast, practical training at the University of Forestry is focused on the pet animals, which represent a lower epidemiological risk. This may explain to some extent the smaller number of students from this University, who had contact with the infection.



Our study revealed a higher seropositivity rate among male students as compared to the female ones, with 32.8% of males testing positive for anti-*C. burnetii* phase II IgG antibodies versus 16.0% of females. This sex disparity is in agreement with our previous observations [9] and suggests that male students might have more intense or prolonged exposure to risk factors during their practical training.

The detection of *C. burnetii* DNA in some blood samples implies that certain infections were in the incubation period or early acute phase. These findings are critical for early diagnosis and prompt treatment to prevent the progression of Q fever.

The study emphasizes the need for improved safety protocols and infection control measures in veterinary training programs. Educating students about the risks and implementing strategies to reduce exposure to *C. burnetii* are essential steps in safeguarding their health.

The present study has some limitations that need to be addressed. First, the research involved a small number of participants. Second, we present results from the serological and PCR assays but do not have data on the associated risk factors. Third, study participants did not have clinical signs and symptoms of Q fever, i.e. all students were clinically healthy and laboratory and imaging studies were not performed. Despite these limitations, this research has its merits. To the best of our knowledge, this is the first study that presents serological evidence of *C. burnetii* infection among veterinary medicine students from Bulgaria. In addition, we performed PCR analysis of the studied serum samples. These are important contributions to the knowledge of this infection in our country and the region of Southeastern Europe (Balkan peninsula).

## 5. Conclusions

In the present study, we presented data on the prevalence of *C. burnetii* infection among a risk group (veterinary medicine students) from Bulgaria. We found high prevalence rates of this pathogen (30.8%) among study participants. This shows that veterinarians in our country are exposed to an increased risk of this infection, which can occur as an acute disease or a chronic infection. All this requires the national health authorities to take adequate measures for the control and surveillance of Q fever among risk groups. In this direction, an important step is to increase the awareness of the veterinary community in our country regarding the measures to prevent Q fever.

**Author Contributions:** Conceptualization, P.G.K., I.T. and M.B.; methodology, P.G.K. and M.B.; formal analysis, P.G.K. and M.B.; investigation, P.G.K., I.T., R.P., B.V., M.B. and P.E.F.; resources, I.T., R.P., B.B.M. and B.C.; data curation, P.G.K., V.D., Y.H., K.T., S.K., K.S. and M.B.; statistical analysis, Y.H.; writing – original draft preparation, P.G.K. and M.B.; writing – review and editing, P.G.K., I.T., M.B., S.P. and P.E.F.; visualization, P.G.K., Y.H. and M.B.; supervision, P.G.K., I.T., M.B. and P.E.F.; project administration, P.G.K. and I.T.; funding acquisition, I.T. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the Local Ethics Committee of National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria (NCIPD-02 / 07 February 2023). The authors have not used artificial intelligence (AI) to create this manuscript.

**Informed Consent Statement:** Written informed consent was obtained from all subjects involved in the study.

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**Conflicts of Interest:** The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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