THE INFECTION RATE OF *COXIELLA BURNETII* IN HUMANS IN THE AGE GROUP OVER 40 YEARS IN THE WESTERN PART OF NORTH MCEDONIA

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Abstract: *Coxiella burnetii* is the causative agent of Q fever, a worldwide zoonosis, and can be misused as a biological warfare agent. *C. burnetii* is classically a strict intracellular, Gram-negative bacterium, that infects a wide range of animals, from arthropods to humans. Cattle, sheep, and goats are the most important source of human infections. Infection in nature is maintained and transmitted by ticks as the principal vector and reservoir. Humans contract *C. burnetii* infection mostly by aerosol in contact with contaminated environs, wind playing an important factor in spreading the infection. The wide distribution of *C. burnetii* contributes to a high resistance of its extracellular small cell variant to environmental conditions. Its intracellular large cell variant, adapted to survive under harsh conditions of phagolysosomes, enables long-term survival and persistence of *C. burnetii*, namely in monocytes/macrophages. Host factors such as underlying disease and cell-mediated immunity play a decisive role in the clinical expression of *C. burnetii* infection. Complete genome analysis of *C. burnetii* will certainly contribute to better understanding of the pathogenesis of *C. burnetii* infection and will improve Q fever diagnosis and immunoprophylaxis. Infection in animals (coxiellosis) is mostly persistent. Infection in humans is often asymptomatic, but it can manifest as an acute disease (usually a self-limited flu-like illness, pneumonia, or hepatitis) or as a chronic form (mainly endocarditis, but also hepatitis and chronic fatigue syndrome). *C. burnetii* infection in pregnant women may result in abortions, premature deliveries, and stillbirths. The objective of this seroepidemiological study was to estimate the prevalence of antibodies to *C. burnetii* and to examine the variation of infection in females and males aged over 40 years, in the western part of North Macedonia. The study was conducted in 274 random people with different symptoms from rural and urban regions of five areas. IgG antibodies against *C. burnetii* were assayed using the ELISA Test. Positive test was based on the Cut-off value, which in this case is over 0.5 optical density (OD). Of 142 female serums which were examined, 38 samples resulted positive (26.76%), and of 132 male serums which were examined, 39 resulted positive (29.54%). As to the percentage of infection in both sexes, there are variations in different regions. The lowest percentage is recorded in Gostivar with 7.5% for males and 13.3% for females, while the highest in Dibra which is 45.4% for males and 42.8% for females in Kercova. The infection percentage in total was high in both sexes and was different against the respective gender based on regions, as well and there is also a connection between the five regions when it comes to the distribution of the infection. The living conditions in the zones covered with the study are poor which represents a predisposing factor in the spread of the infection.
Keywords: *Coxiella burnetii*, Q fever, antibody prevalence, seroepidemiological

1. INTRODUCTION

Q fever has long been considered a rare and regionally restricted disease. In recent years, spectacular advances have been made in the knowledge of this disease and its causative agent, *Coxiella burnetii*. The classification of *C. burnetii* by the Statistic and Epidemiology CDC as a potential bioterrorism agent resulted in the disease becoming reportable in many countries, such as in the United States, which revealed that the disease is more common than previously thought. The recent war in the Middle East (White, B., et al.2013) and research in the tropics (Angelakis, E., et al. 2014; Eldin, C.,et al., 2014) have shown that Q fever may be a very common cause of fever in the intertropical area. Finally, a very large outbreak in the Netherlands has shown that this disease could become a major public health problem (Delsing, C. E., et al. 2010).

Furthermore, knowledge about *Coxiella burnetii* has evolved, with the sequencing of multiple genomes of bacterial strains and their culture in axenic medium. This breakthrough enables genetic transformation and opens a new era. Moreover, redefining the clinical forms of Q fever is necessary, because of a lack of consensus on the distinction between acute Q fever and chronic Q fever (Million & Rault, 2015). This redefinition, by more precisely qualifying the different clinical forms of the disease, will improve the exchange of medical and scientific knowledge about the disease throughout the world.

C. burnetii has a cell wall similar to that of Gram-negative bacteria. However, this small coccobacillus is not stainable with the Gram technique. The Gimenez method is used to stain *C. burnetii* isolated in culture or directly in clinical samples (Gimenez, D. F., 1964). The estimated doubling time of the bacterium is between 20 and 45 h in in vitro cell culture (Angelakis & Rault, 2010). It is an intracellular pathogen, replicating in eukaryotic cells. Its vacuole of replication progressively acquires phagolysosome-like characteristics, such as an acidic pH, acid hydrolysates, and cationic peptides (Voth & Heinzen, 2007). The bacterium actively participates in the genesis of this intracellular vacuole and has several strategies for adaptation to this exceptionally stressful environment. First, *C. burnetii* encodes an important number of basic proteins that are probably involved in the buffering of the acidic environment of the phagolysosome-like vacuole (Seshadri, R., et al. 2003).

Q fever cases have been reported almost everywhere they have been sought, except in New Zealand. The main reservoirs of *C. burnetii* are cattle, sheep, and goats. However, in recent years, an increasing number of animals have been reported to shed the bacterium, including domestic mammals, marine mammals, reptiles, ticks, and birds (Anderson, A., et al. 2013). Birth products contain the highest concentration of bacteria, but *C. burnetii* is also found in urine, feces, and milk of infected animals (Angelakis & Rault, 2010; Guatteo, R., et al. 2006; Rodolakis, A., et al. 2007). Transmission to humans is most frequently due to inhalation of aerosolized bacteria that are spread in the environment by infected animals after delivery or abortion (Angelakis & Rault, 2010).

Most human infections occur after inhalation of infected aerosols of *C. burnetii* (Maurin & Rault, 1999; Tissot-Duppont & Rault, 2008; Parker, N. R., et al. 2006). Infection may occur after direct exposure to infected animals and their products, especially at the time of parturition or slaughtering (Robyn, M. P., et al. 2015; Kaplan, & Bertagna, 1955; Dupont, H. T., et al. 1995). Because *C. burnetii* may persist for prolonged periods in the soil, these aerosols may also be produced long time, after the release of bacteria by infected animals. Moreover, bacterial aerosols can be spread for at least 30 km by the wind (Tissot-Dupont, H., et al. 2004), resulting in Q fever cases far away from the primary contaminated areas. Thus, Q fever cases are often diagnosed in persons with no recent contact with animals.

People may also get sick with Q fever by consuming contaminated, unpasteurized dairy products (Eldin, C., et al. 2013; Rodolakis, A., 2009). Although this mode of contamination has not been proven in humans, ticks may play a role in the transmission of *C. burnetii* infection (Mancini, F., et al. 2014). Rarely, Q fever has been spread through blood transfusion (Saiti & Berxholi, 2015), from a pregnant woman to her fetus (Rault & Stein, 1994), or through sex (Milazzo, A., et al. 2001).

C. burnetii primary infection can manifest itself through a wide diversity of clinical symptoms. After primary infection occurs, *Coxiella* organisms may persist in the host and give rise to subacute endocarditis, chronic hepatitis, and osteoarticular lesions. Persistence may also be related to a prolonged post-infection fatigue syndrome (Marrie, T. J., 1990; Penttila, I. A, et al. 1998).

2. OBJECTIVES

The primary objective of the present seroepidemiological study was to determine the prevalence of infection with Q fever in humans, in the over-40 age group, carried out in the western part of North Macedonia.

3. METHODOLOGY

The study was conducted in the Laboratory of Virology of the Faculty of Veterinary Medicine – Tirana, Albania, using the ELISA Test in humans, a highly sensitive test for the identification of *Coxiella burnetii* antibodies (IgG). The ELISA Kit was imported from the German Firm, SERION. The aim of the study was to identify the IgG (presence of Q fever antibodies). The blood was taken from random people with different symptoms from rural and urban regions. It was collected from Gostivar, Tetova, Kercova, Struga and Dibra areas, in the western part of North Macedonia. The number of people tested was 274, out of which, 132 were males and 142 females. The blood serum was separated by centrifugation at 6000 rpms in 20 minutes. The serum placed in plastic ampoules was kept frozen at -30^{0}C, until its testing. The sera were diluted before the test at a ratio of 1:400, in two steps. The first dilution was done at a ratio of 1:100, then the second dilution, at a ratio of 1:4. They are then incubated for 45 minutes and after the rinsing, the conjugation is added and then other ingredients. In the end the halting solution is added. The incubation time matches the preset criteria for the respective kit. The test was conducted based on the protocol of Serion Firm.

Positivity was based on the Cut-off value, which in this case was over 0.5 optical density (OD). The measurement of OD was done by ELISA reader in 450nm.

The calculation of results (for every controlled serum) was made using the following formula:

$$\mathbf{S/P = \frac{ODsample - OD_{NC}}{OD_{PC} - OD_{NC}}}$$

(NC= Negative Control; PC= Positive Control; OPD sample = OD of the controlled sample)
The assessment of controlled serums is based on the data taken from above formulas having in mind that

S/P ≤ 40% = Negative; 40% - ≤ 50 % suspicious; ≥ 50 % positive

The aim of the study was the prevalence of *Coxiella bunrnetti* antibodies, not the interpretation of the diagnostic results.and not interpret diagnostic outcomes. The data were pooled and processed to determine the percentage (%) of *Coxiella burnetii* antibody presence in general, as well as in the observation and analysis of eventual gender differences regarding the frequency of Q fever. The correlation coefficient between the two variables was also calculated: age and% of Q fever by *Coxiella burnetii*.
During data processing, correlation and regression analysis was performed; namely the determination of correlation coefficients between variables; regression analysis; determination of linear regression coefficients etc.

4. RESULTS

Samples of work with men and women in the over-40 age group

In our study, as mentioned above, 274 people are involved (142 samples from females and 132 samples from males). They were over 40 years old and not all of them lived in rural areas or were in contact with the animals, however, the presence of Q fever antibodies was present in them like those from rural and urban areas. The initial male and female sample data have been separated from region to region (Gostivar, Tetova, Kercova, Struga and Dibra) in the western part of North Macedonia. The serologic examination confirmed the presence of antibodies to *Coxiella burnetii* in almost all zones, though with a different level in different areas and in different genders. In the western part of northern Macedonia, no data are available on the prevalence of Q fever in the human population. Apart from the epidemiological situation of the Q fever in the human population, we have investigated it and in animals in the same regions where people have been observed and we have noticed the presence of the infection with about 15.89% positivity from a total of 1120 examined farm animals serums [23], yet, based on the findings of the foreign authors, we think that the infection of the people comes as a result of the presence of the infections in animals which plays an important role in spreading the cause in the environment, as well as through its airborne distribution.
The results of the study sample in question: region -I, range interval of age group by regions -Xmi (year), total number of tested persons –Ni, numerical frequency of persons infected with *C. burnetii* -Yoi (num) and relative.

Table 1. Data of the working sample divided into five regions with females or males in the age group over 40 years

			FEMALE		
I	Xmi (year)		Ni	Yoi (num)	Yoi (%)
Region	Range interval of age group by regions		Total number of tested persons	Numerical frequency of persons infected with *C. burnetii*	Relative frequency of persons infected with *C. burnetii*
Tetova	>40	40,05	38	8	21.00%
Gostivar		45,05	30	4	13.30%
Dibra		50,05	28	10	35.70%
Kercova		55,05	28	12	42.80%
Struga		60.05	18	4	22.20%
		Total amount	142	38	26.76%

I	Xmi (year)		MALE		
			Ni	Yoi (num)	Yoi (%)
Region	Range interval of age group by regions		Total number of tested persons	Numerical frequency of persons infected with *C. burnetii*	Relative frequency of persons infected with *C. burnetii*
Tetova	>40	40,05	37	13	35.10%
Gostivar		45,05	40	3	7.50%
Dibra		50,05	22	10	45.40%
Kercova		55,05	25	11	44.00%
Struga		60.05	8	2	25.00%
	Total amount		132	39	29.54%

The observed prevalence of males and females infected with the *Coxiella burnetii* has been illustrated in Figure 1.

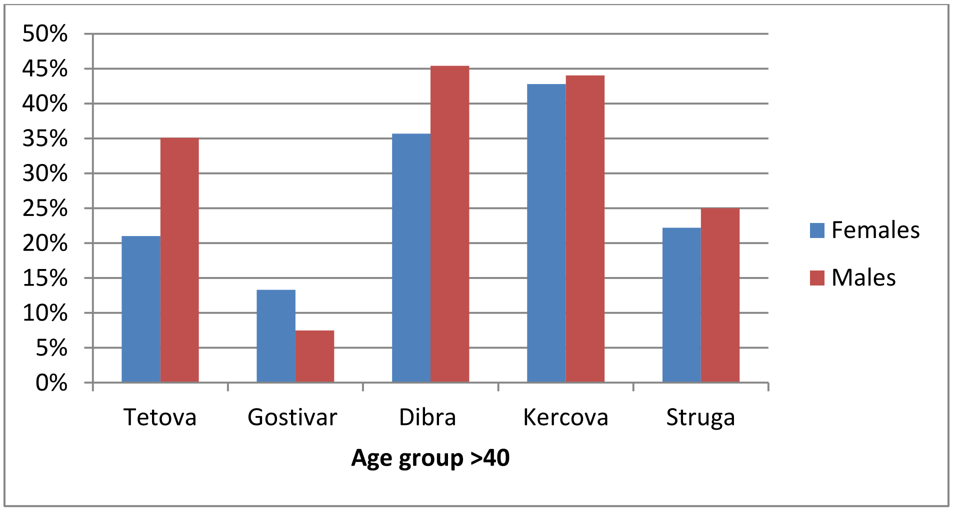

Figure 1. Relative frequency observed in persons infected with Coxiella burnetii for female/male sample

Variation of the frequency of people with *Coxiella burnetii* females or males in the age group over 40 years

Table 2 shows data extracted from table 1, for y_{oif} (%) and y_{oim} (%), referring to x_{mi} (year) values, in the five respective regions with female or male humans, as well as results obtained in their processing based on a linear model function.

Table 2. Variation of the frequency of the infection in both genders

I	Xmi (year)		FEMALE	
			Yoi (%)	Correlation of coefficient (r)
Region	Range interval of age group by regions		Relative frequency of persons infected with *C. burnetii*	Siginificance (p) The equation of linear regression line (y)
Tetova	>40	40,05	21.000%	**r = 0,42**
Gostivar		45,05	13.30%	
Dibra		50,05	35.70%	**0.025<p<0.05**
Kercova		55,05	42.80%	
Struga		60,05	22.20%	**y = 0,0054x - 0,0493**

I	Xmi (year)		MALE Yoi (%)	Correlation of coefficient (r)
Region	Range interval of age group by regions		Relative frequency of persons infected with *C. burnetii*	Siginificance (p) The equation of linear regression line (y)
Tetova		40,05	35.10%	**r = 0,16**
Gostivar		45,05	7.50%	
Dibra	>40	50,05	45.40%	**0.025<p<0.05**
Kercova		55,05	44.00%	
Struga		60,05	25.00%	**y = 0,0033x + 0,1508**

For couples of variables [X_{mi} (year), Y_{oi} (%)] and [X_{mi} (year), Y_{oi} (%)], of table 2, are determined correlation coefficients $\mathbf{r_f}$ **=0,42** and $\mathbf{r_{m=}}$ **0,16** both worth of significance 0,05>p>0,025 [4,10,19]. The equations of linear regressions lines resulted:

$$\mathbf{Y_{ef} = 0,0064x - 0,0493 \ (1)}$$
$$\mathbf{Y_{em} = 0,0033x + 0,1508 \ (2)}$$

Chart 2 ilustrates the position of (1) and (2).

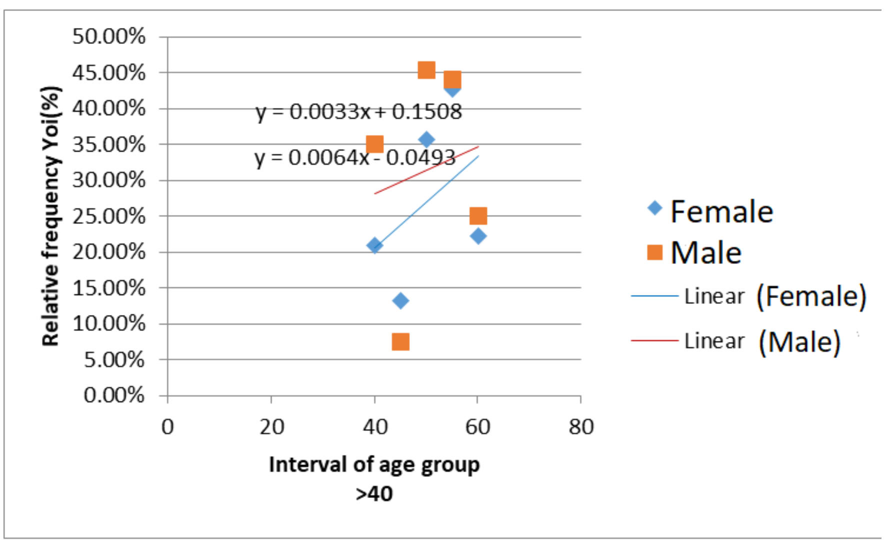

Chart 2. Positions of the straight-linear regression to expected relative frequencies

5. DISCUSSION

As we can see from Table 1 and Figure 1, the data presented reveals the presence of the Q-fever in people in the western part of North Macedonia. This infection was present in both males and females, with a relative frequency of 29.54% and 26.76% respectively. The difference between genders, as can be seen, in this case was 2.78%, so the males had a higher infection rate.

The infection rate was different against the respective gender based on regions as well. In terms of males, the highest infection rate of 45.5% was in the region of Dibra. Meanwhile, in other regions this percentage was a little lower; in Kercova it reached 44%, in Tetova 35.1% and in Struga 25%. It should be noted that in the region of Gostivar, the infection rate in males was 7.5%, i.e. lower than any of the other regions.

In terms of females, the prevalence of *C. burnetii* infection was also high, which in Kercova reached up to 42.8%; compared to the other regions, it was 7.1% higher than in the Dibra region and twice as high as the infection observed in the Tetova region, but also many times higher than in the Gostivar region (nearly four times) and also twice as high as in the Struga region. In this region, in addition to the above mentioned level, we found that even in the age group of 20-40 years, the infection rate was very high, reaching 62.5%, whereas in the other regions the infection rate in this age group was almost half as the infection observed in Kercova (Saiti, I, et al. 2017). In this case, we think that the Kercova region is the most affected, not only that there were high levels of infection in farm animals (Saiti & Berxholi, 2015), but also the fact that this age group is more concerned with the care of these animals, especially in rural areas. According to Maurun and Raoult (1999) living close to sheep herds is directly linked to the increase of the risk of being exposed to the infection with the Q fever, since one of the predominant ways of contamination in people is breathing the infected air (an experience from the burst of the Q fever in people in Germany in 1996). This is more emphasized in women than in men, especially with regard to small farm animals (sheep and goats) during the period of births, which based on research, is one of the most common ways of the spread of the infection in people. This fact of ours is also supported by other authors (Heinze, R. A., et al. 1999; Tissot-Dupont, H., et al. 1992), who point out the fact that the highest infection rate in people can be noticed during the period of birth of small farm animals. We are not claiming that this particular datum in the concrete case is directly related to the percentage of seasonal infection, but we see this phenomenon mentioned by the above-stated authors as having indirect relation to the infection of female persons, because females in this region are more closely related to animals, and they, based on our data (personal communication with women in this region) help animals with their difficult births and as a result the chances to get the infection under these circumstances are much higher. This is one of the possible factors, in our opinion, that causes the higher infection rates in this particular age group. This phenomenon can probably happen due to other factors as well, such as social and ecologic ones.

Regarding the Dibra region, the data shows that despite the fact that in this region the number of examined individuals, in the age group of over 40, for both male and female differs for about only six samples, and the number is not considerable, it turns out that we have the same infection in both sexes (with 10 positive samples for each sex). It appears that, the various factors that play a role in transmitting the infection to humans do not have a significant impact.

In the Tetova region there is a more pronounced difference of infection in males, by about 14.1% higher than females.

The Gostivar region results with the lowest infection rate of all the other regions for both sexes. In males, the infection rate is about 7.5%, while in females it is about 13.3%, i.e. approximately in the ratio of 1:2. This data obtained by us is inconsistent with the data provided by foreign authors, namely the French ones (Tissot-Dupont, H., et al. 1992), who, based on their conducted studies, have found that the ratio of infected males and females is almost 1:1, although, the author points out that there are times when the ratio is 1:2 in favor of men. We think that in order to clarify this point, it is necessary for other studies to be undertaken, setting off from the epidemiological point of view.

In the Struga region, prevalence of antibodies was higher in males than in females, with a difference of 2.8%. This difference, in our opinion, which is also supported by foreign authors, is that the level of infection with *C. burnetii* by age and sex may vary from one area to another, depending on where the infected animals predominate, from which patients become infected as well as opportunities for exposure to these infected animals (Maurin & Rault, 1999). In this region, the highest infection rate was observed in the age group of 20-40, in males with 37.5% and in females with 33.3% (Siti & Berxoli, 2015). This data is almost consistent with data by foreign authors (Maurin & Rault, 1999)., who point out that Q fever appears unexpectedly and is more common in men of active age from 30-60 years.

Based on the study of the data from the table, we can see that the infection in females and males has no significant difference between them. Thus, in males the total infection rate was about 29.54%, while in females it was about 26.76%, which is a difference of 2.78%. This data that comes from our study is inconsistent with the statistical data of CDC in the United States, which mentions that the rate of infection in the male sex is twice as high as in the female. This phenomenon is probably due to the fact that other social or ecological factors also interfere here. However, based on the above table, it turns out that the infection in men is higher.

According to Chart 2 (Table 2) we can see that in the age group of over 40, individuals of both genders in all the regions were infected with *Coxiella burnetii*. When analyzing the linear regression lines, we find positive directions in both cases, so with the increase of the controlled number, the frequency of the infected number will also increase, but in the distribution of the individual cases from the average or line in males, we see that the cases are far from the line. This shows that the connection between the frequency of the infection and the number of controlled cases in this age group is weak, so these two variables have very little correlation with each other. Also, in females, the individual cases are far from the line, but not as much as in males. This shows that here there is also no close connection between the frequency of the infection and the number of examined cases. From this, we can see that with the increase of the controlled number, the increase of the frequency of infection is slightly affected, which is indicated by the correlation coefficient for females, which is 0.4 (a weak but significant coefficient), whereas in males the correlation is 0.16 (insignificant correlation), indicating no dependence between these two variables.

6. CONCLUSION

Based on our serological research, *Coxiella burnetii* infection was highly present in both sexes and with an approximate frequency between them. The infection percentage was different against the respective gender based on regions. With regard to males, we point out that the infection rate was very high in the region of Dibra, while in females the highest infection rate was detected in the Kercova region. We concluded that there is a connection between the five regions when it comes to the distribution of the Q fever infection in people. The living conditions in the zones covered with the study are poor which represents a predisposing factor in the spread of the infection. Our data are based on the analyses provided by other researchers who say that *Coxiella burnetii* infection in humans is an illness mainly present in the developing countries.

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