VETERINARSKI ARHIV 93 (5), 495-502, 2023 DOI: 10.24099/vet.arhiv.1866

Coxiella burnetii occurrence in dairy herds in Gabrovo Region, Bulgaria, evaluated by serological and molecular analyses of bulk-tank milk samples

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SIMEONOV, K. B., P. D. GENOVA-KALOU: *Coxiella burnetii* occurrence in dairy herds in Gabrovo Region, Bulgaria, evaluated by serological and molecular analyses of bulk-tank milk samples. Vet. arhiv 93, 495-502 2023.

ABSTRACT

This study investigated the occurrence of *Coxiella burnetii* in dairy cattle, goat herds and sheep flocks in the Gabrovo Region, central Bulgaria, to identify the potential sources of *Coxiella burnetii* infection diagnosed in veterinarians and farm workers in this region. To detect infection on livestock farms, we tested bulk-tank milk (BTM) for the presence of antibodies and/or the *Coxiella burnetiid* genome using ELISA and PCR, respectively. A total of 81 herds were tested, including 23 dairy cattle herds, 43 sheep herds, 9 goat flocks, and 6 mixed flocks (sheep and goats). By ELISA, antibodies against *Coxiella burnetii* were detected in 30.4% of the BTM tested samples from cattle farms, 60.4% of the sheep farms, and 11.1% of the goat BTM samples. The results were inconclusive in 6.98% of the tested sheep milk samples and 11.1% of the goat milk samples. There was a statistically significant correlation between the herd size and the ELISA S/P % values on the dairy cattle farms. Excretion of the pathogen in milk was detected by PCR in 9 out of 67 BTM samples, including 5 out of 19 cattle BTM samples, 3 out of 39 sheep BTM samples and 1 out of 5 goat BTM ones. The results indicate that *C. burnetii* infection is widely prevalent in the region, which calls for adequate control and prophylactic measures to reduce the health risks from the transmission of this zoonosis to humans.

Key words: *Coxiella burnetii*; Q fever; bulk tank milk; dairy cattle, sheep and goats; ELISA; PCR.

Introduction

Human or animal Q fever has been described in almost every country in Europe, including Balkan countries (SAITI et al., 2017; CHOCHLAKIS et al., 2018; DEBELJAK et al., 2018). In Bulgaria, Q fever is endemic in many regions, with occasional epidemic outbreaks, reportedly affecting hundreds of people (SERBEZOV et al., 1999; KAMENOV and TIHOLOVA, 2004). Most commonly, however, there have been smaller outbreaks or sporadic cases of infected Individuals, with year-to-year variation (GENOVA-KALOU et al., 2019). Moreover, the exact number of infected people is difficult to

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ISSN 0372-5480
Printed in Croatia

determine, as many remain undiagnosed due to the lack of symptoms or the presence of only a mild clinical manifestation. In addition, even in cases of pronounced symptoms, Q fever is not always considered as a possible cause since the clinical signs overlap with those of other diseases, and thus it often remains underestimated (ANDERSON et al., 2013). Domestic ruminants (cattle, sheep, and goats) are the major source of infection in humans (ANGELAKIS and RAOULT, 2010). These animals release large quantities of the pathogen with birth by-products, during abortion or normal parturition, therefore, endemic outbreaks of Q fever in humans are most often observed around the period of mass lambling (DEBELJAK et al., 2018). Q fever was considered predominantly an occupational hazard for those directly engaged in the management and handling of such animals, as well as for those working in animal processing plants (EFSA, 2010; RAHAMAN et al., 2020). Recent testing has found active infections in livestock farmers and veterinarians from the Gabrovo Region, based on positive PCR results and/or IgM seropositivity in ELISA (GENOVA-KALOU et al., 2021). The infection was also detected in herds and flocks of ruminants in farms in this region in previous years (Simeonov, unpublished data). Therefore, it was necessary to test as many dairy cattle, sheep, and goat farms as possible, to identify exposure to sources of human Q fever and to evaluate the current status of the disease in the area.

In Bulgaria, the *C. burnetii* infection is monitored by a National Program for the prevention, surveillance, control, and eradication of animal diseases, including zoonoses, which includes serological testing of individual blood samples, collected from domestic ruminants. However, this approach has the disadvantage that only a limited number of randomly selected animals from a relatively small number of livestock farms in a region are tested. Hence, the probability of some infected animals in a herd or flock remaining undetected is real. At the same time, collecting blood samples from individual animals is labour- and time-consuming, and laboratory diagnosis incurs considerable costs for consumables due to the high price of diagnostic kits. As a result, diagnosis at a herd/flock level, rather than at the individual animal level, to monitor the circulation of *C. burnetii* has been suggested as more suitable for the detection of outbreak sources (EFSA, 2010).

A comparatively low-cost alternative to serological blood testing, which is useful and easily accessible for large-scale epidemiological investigation is the analysis of bulk-tank milk (BTM). BTM testing has the advantage of detecting herds/flocks that have been previously infected with *C. burnetii* in a single sample (MUSKENS et al., 2011), it provides a preliminary evaluation of the herd status, allows ease of sampling, and reduces costs. Some authors (RODOLAKIS et al., 2007) even suggest that the detection of antibodies in milk could be more sensitive than in the serum. In addition, PCR testing of BTM makes it possible to detect herds with animals that are active shedding the pathogen and thus, to conduct a sanitary assessment of milk for human consumption (HENGL et al., 2017; PEXARA et al., 2018).

The aim of this study was to perform ELISA and PCR analyses of bulk-tank milk samples from farms with dairy cattle, sheep herds and goat flocks, and to identify the ones that pose a high zoonotic risk as a potential source of human infection, as well as to estimate the prevalence of *C. burnetii* in the Gabrovo Region (Bulgaria).

Materials and methods

Study area. The Gabrovo Region is located in central northern Bulgaria and covers an area of 2023 km^2. It includes 4 administrative municipalities: Gabrovo, Dryanovo, Sevlievo and Tryavna. The region is semi-mountainous; it is suitable for animal husbandry, which constitutes an important part of the rural economy. There are 331 registered cattle farms, 315 goat farms, and 469 sheep farms in the region. The total number of animals in them is estimated at over 24 000, including 7675 cattle, 12,770 sheep and 4057 goats (BFSA, 2021). Semi-extensive production is predominant.

Study design. The study was carried out from April to June 2020. It included a random selection of 23 dairy cattle herds, 43 sheep herds, 9 goat flocks, and 6 mixed ones (sheep and goats) from 51

settlements in the region. Samples were collected specifically for this investigation by authorized veterinarians following standard procedures. The sterile plastic containers containing specimens were delivered for testing to the Laboratory for Chlamydial and Rickettsial Diseases, of the National Diagnostic and Research Veterinary Medical Institute, Sofia. Two mL of each sample were defatted by centrifugation in Eppendorf tubes at 14 000 rpm for 2 min after which the top fatty layer was removed mechanically. The milk serum was transferred to another Eppendorf tube and used directly in ELISA for detection of antibodies against *C. Burnetii,* or was frozen at -20°C until use. The pellet, containing somatic cells, was washed twice with PBS, at pH 7.4, suspended in 200 mL of PBS, and used for DNA extraction.

ELISA. A commercial ELISA ID Screen® Q Fever Indirect Multi-species Kit (ID.vet, France) was used for the detection of *C. burnetii* antibodies in bulk-tank milk, following the manufacturer's instructions. The optical density (OD) was read using an LKB ELISA reader; the S/P % ratio was calculated and the results were interpreted according to the manufacturer's instructions as follows: negative (S/P%≤30), doubtful (30≤S/P%≤40) and positive, when the S/P% >40. To obtain a more detailed assessment of the seroprevalence among the lactating animals in the herds/flocks, the positive milk samples were additionally categorized into two semi-quantitative classes, respectively positive (+; 40<S/P%≤100) and strongly positive (++; S/P%>100).

PCR. A conventional PCR assay was performed to detect the presence of *Coxiella burnetii* genome in milk samples. All samples that gave positive ELISA results were tested, as well as some of those that gave negative ELISA results. DNA was extracted from somatic cells using the IndiSpin®Pathogen Kit (INDICAL Bioscience GmbH, Germany) according to the manufacturer's instructions, and stored at -20°C until PCR analysis. Each DNA sample was tested by PCR with primers Trans ½, targeting the repetitive transposon-like region of *C. burnetii* using the amplification protocol as described previously (BERRI et al., 2000). All dairy farms in which the BTM sample tested positive for the presence of antibodies or genome of *C. burnetii* were considered as infected. According to the regulatory requirements in Bulgaria, the responsible public health authorities (Regional Health Inspection, RHI) were informed, and respective measures were undertaken, including serological (IgM/IgG), or PCR testing of blood samples from veterinarians and farm workers (GENOVA-KALOU et al., 2021).

Statistical analyses. The mean S/P% ELISA values for the three groups of farms (with negative, inconclusive, or positive results) were analyzed using the nonparametric Kruskal–Wallis Test, and the differences were considered statistically significant at the level of $P < 0.05$. A linear model of correlation was performed to determine if a relationship between ELISA S/P% values obtained and the herd size existed. The correlation coefficient R was estimated using IBM® SPSS® Statistics software.

Results

Thirty-five (43.21%) out of a total of 81 milk samples analyzed by ELISA were positive for the presence of antibodies, and 5 (6.17%) gave a doubtful (30≤S/P≤40) result. The presence of seropositive lactating animals was detected on 7 out of the 23 tested cattle farms (30.4%). Twenty-six positive (60.47%) and 3 samples with doubtful results (6.98%) were found among the 43 BTM tested samples from sheep flocks. In the tested goat herds (n-7), 1 BTM sample was positive and 1 sample showed an equivocal result (11.1% each). Also, one positive and one doubtful result were obtained after testing of 6 BTM samples from mixed (sheep and goats) flocks. The estimated S/P% values after ELISA testing of milk samples from cattle showed a wide variability (ranging from 2.61 to 253), and the comparison of the mean S/P% values of the tested samples with negative (6.50±2.57) and positive (164.05±74.69) results showed a statistically significant difference between the two groups, evaluated by T-test ($P<0.05$). In addition, a strongly positive correlation ($R=0.615$) between S/P% values, estimated by ELISA testing of the BTM samples, and the cattle herd size

was observed, which was statistically significant (P=0.002). In the sheep samples, the mean S/P% value was 48.33±32.89, and in the BTM samples from goat herds and mixed (sheep and goat) flocks the mean values were 39.52 ±15.58 and 27.33 ±15.51, respectively.

The presence of the pathogen in the milk was detected by PCR in 9 out of a total 67 BTM samples tested, including 5 out of 19 cattle BTM samples, 3 out of 39 sheep BTM samples, and 1 out of 5 goat BTM samples. The results of the serological and molecular assays of BTM from sheep flocks, goat and cattle herds are shown in Table1.

C. burnetii genome was not detected in the tested BTM from mixed flocks (sheep and goats). There were also no positive PCR results among the tested BTM from seronegative herds/flocks of both large and small ruminants.

Table 1. Distribution of *C.burnetii* positive and negative herds with respect to results obtained from serological and molecular analyses of bulk-tank milk (BTM)

Livestock	BTM ELISA		Number of herds/flocks	
			BTM PCR	
Cattle	Negative*		16	0
	Doubtful		0	0
	Positive(total)		7	5
		positive	2	1
		strongly positive	5	4
Sheep	Negative		14	0
	Doubtful		3	0
	Positive (total)		26	3
		positive	20	3
		strongly positive	6	0
Goats	Negative		7	0
	Doubtful		1	0
	Positive (total)		1	1
		positive	1	1
		strongly positive	0	0
Mixed herds	Negative		5	0
	Doubtful		1	0
	Positive (total)		1	0
		positive	1	0
		strongly positive	0	0

"Negative" indicates that the sample-to-positive control (S/P) ratio was ≤30; "doubtful" indicates 30<S/P≤40; "positive" indicates 40<S/P≤100; "strongly positive" indicates S/P≥100.

Discussion

The epidemic outbreaks or sporadic cases of Q fever in humans are directly associated with the presence of infected animals that shed the pathogen in the environment (EFSA, 2010). Thus, the prevention of human infection relies mainly on the active surveillance of ruminant herds/flocks and the detection of infected ones. In this study, we tested BTM from dairy cattle herds, and sheep and goat flocks on livestock farms to understand the situation regarding coxiellosis in the Gabrovo Region better, and to assess the zoonotic risk the animals pose as a source of infection in humans. The present survey is the first epidemiological report, summarizing the incidence of *C. burnetii* infection in dairy farms in the region, as well as the first one based on the investigation of BTM samples in Bulgaria.

The results of examinations revealed that 43.21% of the total 81 ELISA tested BTM samples had antibodies against *C. burnetii*, and positive cases were detected in the herds/flocks of the three species of ruminants surveyed. Due to the lack of information on the prevalence of *C. burnetii* at the herd/flock level, the obtained results cannot be compared, concerning the extent of infection in other regions of Bulgaria. Nevertheless, the results considerably exceed the overall data about the *C. burnetii* seroprevalence in the ruminant livestock populations in Bulgaria published in recent years, based on serological testing of individual blood samples performed within the framework of the national surveillance program (European Food Safety Authority and The European Centre for Disease Prevention and Control, 2021).

Twenty-five percent (25%) of the tested BTM samples from dairy cattle farms contained antibodies against *C. burnetii*, indicating past or present infection in the herds. Using the same diagnostic approach, PAIBA et al. (1999) described serological evidence for *C. burnetii* infection in 21.5% of cattle herds from England and Wales. BORODUSKE et al. (2017) also reported a lower prevalence in Latvia. On the other hand, similar or higher prevalences have been described for Denmark, 79.2% (AGGER et al., 2010), the Netherlands, 78.6% (MUSKENS et al., 2011), northern Spain, 66.9% (ASTOBIZA et al., 2012), Poland, 45.5% (SZYMAŃSKA-CZERWIŃSKA et al., 2019) and some Central and East European countries, 93.78% (DOBOS et al., 2020). In addition, the estimated mean ELISA S/P% value for the cattle BTM samples was relatively higher (54.45±66.71) than those obtained from testing the sheep and goat BTM samples, 48.33±32.89 and 39.52±15.58, respectively. Interestingly, to a large extent, this was due to the significantly higher levels of antibodies found in the milk from only 5 of the 24 cattle farms investigated, four of them being of the industrial type, with a large number of animals (from 358 to 730). A significant ($P<0.05$) association between the herd size and the BTM antibody levels was confirmed. Other researchers have also reported that antibody positivity is directly proportional to the increase in herd size (McCAUGHEY et al., 2010; RYAN et al., 2011; AGGER et al., 2013; VAN ENGELEN et al. 2014; ANASTÁCIO et al., 2016). On the other hand, ASTROBISA et al. (2012) did not observe such a correlation, and others have even found an inversely proportional relationship (NOKHODIAN et al., 2017). Since S/P% is an indicator of the *C. burnetii* prevalence within herds, and depends on the percentage of seropositive animals in each herd (TAUREL et al., 2012), the active circulation of the pathogen in the above-mentioned large farms is suggested. The maintenance of infection on large-scale farms could be attributed to the higher animal density and intensive reproduction, with a higher number of recently calved cows, which shed the pathogen. Our PCR investigations also confirmed the higher level of shedding *C. burnetii* in the cattle population, with 5 out of 19 cattle BTM samples resulting positive. This is not surprising, since in cattle, the longest duration of excretion is observed (up to several months after parturition) compared to sheep and goats (RODOLAKIS et al., 2007; ASTOBIZA et al., 2010; RADINOVIĆ et al., 2013). In addition, reproduction in cattle is a continuous process and in all herds, there are recently calved cows throughout the year. Conversely, in small ruminants, the intensive release of the pathogen into the milk of infected animals can be expected

during the months of December-March, which is the period of mass lambing. In our study, most of the BTM samples from sheep and goats arrived at the laboratory in June. This may be one of the reasons why the presence of the *C. burnetii* genome was only found in 4 of the 48 BTM samples tested, although a high proportion of them were antibody positive. However, the presence of infected herds of ruminants is important from an epidemiological point of view, as goats and sheep are the species most commonly involved as possible sources of human infection (HATCHETTE et al., 2001; MCQUISTON and CHILDS, 2002).

The positive samples originated from herds/flocks located in the four municipalities in the region. This indicates the diffuse spread of infection. Considering the high zoonotic potential of *C. burnetii* infection, management measures should be taken to control the infection on farms and prevent its transmission to people.

Acknowledgments

This study was supported by the Bulgarian National Science Fund under Grant No KP-06-N33/3/2019 with the 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 particularly dangerous infectious agents causing epizootic and epidemiological outbreaks on the territory of the Republic of Bulgaria", and partially funded by the National Diagnostic and Research Veterinary Medical Institute (Project No 1.54/2019).

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Received: 27 January 2022
Accepted: 18 February 2022

SIMEONOV, K. B., P. D. GENOVA-KALOU: Pojavnost bakterije *Coxiella burnetii* u mliječnim stadima u pokrajini Gabrovo, Bugarska, ustanovljena serološkim i molekularnim analizama uzoraka mlijeka iz cisterni. Vet. arhiv 93, 495-502 2023.

SAŽETAK

U ovom je radu istraživana pojavnost bakterije *Coxiella burnetii* u stadima mliječnih krava, koza i ovaca u pokrajini Gabrovo, središnja Bugarska. Rezultati bi trebali pomoći pri otkrivanju potencijalnih izvora zaraze navedenom bakterijom u veterinara i poljoprivrednih radnika u tom području. Kako bi se otkrila infekcija na stočnim farmama, mlijeko iz velikih spremnika (BTM) testirano je na prisutnost protutijela i/ili genoma bakterije *Coxiella burnetii* primjenom testa ELISA i PCR-om. Testirano je ukupno 81 stado, uključujući 23 stada mliječnih krava, 43 stada ovaca, 9 stada koza i 6 mješovitih stada (ovce i koze). Testom ELISA otkrivena su protutijela na bakteriju *Coxiella burnetii* u 30,4% uzoraka iz BTM-a s farmi krava, 60,4% s farmi ovaca i 11,1% uzoraka BTM-a s farmi koza. Pouzdani zaključci nisu mogli biti doneseni u slučaju 6,98% uzoraka mlijeka ovaca i 11,1% uzoraka mlijeka koza. Uočena je statistički znakovita korelacija između veličine stada i postotne vrijednosti ELISA S/P u mliječnih krava. Izlučivanje patogena u mlijeko otkriveno je PCR-om u 9 od 67 uzoraka BTM-a, uključujući 5 od 19 uzoraka mlijeka krava, 3 od 39 uzoraka mlijeka ovaca i 1 od 5 uzoraka mlijeka koza. Rezultati su pokazali da je infekcija bakterijom *C. burnetii* široko rasprostranjena u pokrajini Gabrovo, što zahtijeva odgovarajuće mjere kontrole i profilakse kako bi se smanjili zdravstveni rizici od prijenosa ovog zoonotskog uzročnika na ljude.

Ključne riječi: *Coxiella burnetii*; Q-groznica; cisterne; mliječna goveda; ovce i koze; ELISA; PCR