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■ Prevalence of *Coxiella burnetii* antibodies in bulk milk and blood serum and associations with reproductive indices in cow dairy herds of Central and Northern Greece

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■ Επιπολασμός αντισωμάτων έναντι της *Coxiella burnetii* στο γάλα και στον ορό και συσχέτιση με αναπαραγωγικούς δείκτες σε εκτροφές αγελάδων γαλακτοπαραγωγής της Κεντρικής και Βόρειας Ελλάδας

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ABSTRACT. For the first time in Greece, we investigated the prevalence of *Coxiella burnetii* antibodies in milk and sera from dairy cattle herds located at central and northern parts of the country. Eighty herds were initially voluntarily enrolled in the study and a bulk milk sample from each farm was assayed by ELISA for *C. burnetii* antibodies. According to antibody titre, herds were classified into 5 categories: negative and grades 1,2,3 and 4 (ascending scale). To assess the prevalence within farms, two herds from each category were selected and blood samples were collected for antibody assessment. In these herds, some reproductive indices were compared between farms; in addition, comparisons were made in paired seropositive and seronegative animals from one grade 3 herd. Twenty three herds (35%) were found positive, 21 being in categories 3 and 4. The prevalence of seropositive animals between herds varied from 4.9 to 46.3%, even from farms initially characterized as negative, some positive animals were detected. Between farms, no differences were detected in the abortion rate or in the mean number of artificial inseminations (AI) per pregnancy. Some differences were found in other reproductive indices that were impossible to be biologically interpreted under the light of *C. burnetii* level of infection. From the results presented here, we infer that *C. burnetii* infection is likely asymptomatic in dairy cows causing minimal –if any- economic losses to farmers. However, since the disease is a zoonotic one, its spread can easily occur, a systematic surveillance, in all ruminant species, for the restriction or eradication of the disease should be undertaken in national level.

Keywords: *Coxiella burnetii*, antibodies, reproduction, cow

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ΠΕΡΙΛΗΨΗ. Στην εργασία αυτή ερευνήθηκε για πρώτη φορά στην Ελλάδα το ποσοστό προσβολής (επιπολασμός) από *C. burnetii* σε αγελάδες γαλακτοπαραγωγής. Ογδόντα εκτροφές αγελάδων της βόρειας και κεντρικής Ελλάδας συμμετείχαν οικειοθελώς στην έρευνα. Δείγματα γάλακτος συλλέχθηκαν από τη δεξαμενή γάλακτος της μονάδας και εξετάστηκαν για την παρουσία αντισωμάτων έναντι της *C. burnetii* με τη μέθοδο της ELISA. Αναλόγως του τίτλου των αντισωμάτων, οι μονάδες κατατάχθηκαν σε 5 κατηγορίες: αρνητικές, 1,2,3 και 4. Για να ελέγξουμε το βαθμό προσβολής σε επίπεδο εκτροφής, επιλέχθηκαν 2 μονάδες από κάθε κατηγορία από τα ζώα των οποίων συλλέχθηκαν δείγματα αίματος τα οποία εξετάστηκαν, όπως και τα δείγματα γάλακτος, για παρουσία αντισωμάτων. Στις μονάδες αυτές έγινε σύγκριση ορισμένων αναπαραγωγικών δεικτών μεταξύ οροθετικών και οροαρνητικών μονάδων. Επιπλέον, σε μια ομάδα κατηγορίας 3, συγκρίθηκαν οι αναπαραγωγικοί δείκτες σε ζεύγη οροαρνητικών - οροθετικών ζώων. Από την εξέταση των δειγμάτων γάλακτος, βρέθηκαν συνολικά 28 θετικές μονάδες (35%), από τις οποίες οι 21 ανήκαν στις κατηγορίες 3 και 4. Το ποσοστό των οροθετικών ζώων στις εκτροφές παράλασσε από 4.9% έως και 46.3%, ενώ οροθετικά ζώα ανιχνεύθηκαν και σε εκτροφές οι οποίες, με βάση την εξέταση του γάλακτος, είχαν αρχικώς χαρακτηριστεί ως αρνητικές. Δε βρέθηκαν διαφορές στα ποσοστά αποβολών και στον απαιτούμενο αριθμό ΤΣ ανά εγκυμοσύνη. Διαφορές εντοπίστηκαν σε διάφορους αναπαραγωγικούς δείκτες, όμως αυτές μάλλον αποτυπώνουν διαφορετικές αναπαραγωγικές πρακτικές και δε θα έπρεπε να αποδοθούν στην προσβολή από *C. burnetii*. Τα αποτελέσματα της παρούσας μελέτης δείχνουν ότι η προσβολή των αγελάδων γαλακτοπαραγωγής από *C. burnetii* είναι μάλλον υποκλινική ή ασυμπτωματική και προκαλεί ελάχιστες – ίσως καμία- οικονομικές απώλειες στον κτηνοτρόφο. Με δεδομένο, όμως, ότι η νόσος μεταδίδεται στον άνθρωπο (ζωοανθρωπονόσος) και η διασπορά της μπορεί να γίνει εύκολα, θεωρούμε ότι είναι επιβεβλημένη η διενέργεια, σε εθνικό επίπεδο, συστηματικής διερεύνησης του βαθμού προσβολής σε όλα τα είδη των μηρυκαστικών, με σκοπό τη λήψη μέτρων για τον περιορισμό ή/και την εκρίζωσή της.

Λέξεις ευρετηρίασης: *Coxiella burnetii*, αντισώματα, αναπαραγωγή, αγελάδα

Introduction

Coxiella burnetii is an obligatory intracellular, Gram negative, very resistant, rickettsial microorganism that replicates in host monocytes and macrophages. It was first reported in 1938 by two independent groups; the organism was later named after the family names of the two senior researchers, Cox and Burnet (Burnet and Freeman 1937, Cox 1938). The organism is endemic all over the world except New Zealand (Hilbink et al. 1993) and *C. burnetii* infections have been reported to a wide range of hosts such as domesticated and wild mammals, birds and arthropods (Maurin and Raoult 1999, Hirai and To 1998). The life cycle of the bacterium has two forms –both of them are infectious- the large cell variant (LCV) and the spore-like small variant (SCV), which is extremely stable to the environment being able to survive for years and, having very small size, it can be transferred by the winds for kilometers (Miller et al. 2006). These properties made *C. burnetii* to be considered as potential agent for biological weapon or bioterrorism and it has been classified as a category B agent (Oyston & Davies 2011).

The shedding route of the microorganism from the infected host includes the milk, the urine, the feces and, mainly, the fetal fluids and membranes which could be heavily contaminated with up to 10^9 microorganisms per gram (Fournier et al. 1998). However, the shedding route of *C. burnetii* varies between

domestic ruminant species; in cows and goats, *C. burnetii* is almost exclusively shed into the milk, whereas ewes excrete the bacteria mostly in milk and feces (Rodolakis et al. 2007). In farm ruminants, *C. burnetii* can induce reproductive failures, such as abortions, delivery of weak newborns, retention of fetal membranes, endometritis and reduced fertility, with the clinical signs being more prominent in small ruminants than in the bovine (Van der Brom and Vellema 2009, Rodolakis et al. 2007). Ruminants are the main reservoir from which humans are infected by *C. burnetii* that gives the ubiquitous zoonosis called Q fever. Usually, the disease has non-specific clinical manifestation, with an onset as a flu-like febrile infection accompanied by severe headache. Atypical pneumonia with non-productive cough and chest pain is very common, but acute infections can cause meningo-encephalitis, pericarditis, thrombophlebitis, arthritis and pancreatitis, with a mortality rate up to 2% (Gikas et al. 2010, Mazokopakis et al. 2010, Hartzell et al. 2008).

In ruminants, the prevalence of the disease varies between countries; for example seropositivity in Korean, Canadian and Danish dairy herds was 25.6%, 67% and 59%, respectively, while, in the USA, this figure goes from 1 up to 94% depending on the state and the method used (Agger et al. 2010, Kim et al. 2005, Kim et al. 2006, Lang 1988).

While we know that Q fever is endemic in some parts of our country (Pape et al. 2009, Antoniou et al. 1995, Tselentis et al. 1995), very little is known on the prevalence of the disease in Greek ruminant population; only Pape and co-workers studied the prevalence of *C. burnetii* in small ruminants in northern Greece (Pape et al. 2009).

Hence, the aim of this study was to assess, on the basis of antibodies in bulk milk, the prevalence of *C. burnetii* infection in cattle dairy farms in central and northern Greece (the area with the highest concentration of dairy farm) and then to correlate some basic reproductive indices with seroprevalence.

Materials and methods

Eighty farms from the regions of Macedonia, Thrace and Thessaly were voluntary enrolled to the study. Each day 20 samples were collected in sterile 100ml containers, from the outlet of the milk tank; to minimize cream concentration, the mixer was stopped 15 to 30 min prior to collection. Samples were transferred ice-cold in the lab, they were centrifuged to remove fat and the non-fat fraction was stored at -20°C until tested for antibodies.

Milk antibody titres against *C. burnetii* were assayed using an initial 1/20 dilution, by a commercial indirect ELISA kit (LSIVET RUMINANT, Milk/serum Q fever, INRA, Lissieu, France) according to the manufacturers instructions. The antibody titre was expressed as S/P value X100, according to the equation:

$$S/P = (OD \text{ sample} - OD_{NC}) / (OD_{PC} - OD_{NC})$$
, where S=sample, P=positive, OD=optical density, NC = negative control, PC = positive control.

A titre ≤ 30 was considered negative, while positive samples were categorized into 4 grade scale according to their titres: 30 to 100 grade 1, 100 to 200 grade 2, 200 to 300 grade 3 and > 300 grade 4.

On the basis of milk antibody titres, two farms from each category were selected to screen the seroprevalence. These farms were located at the regions of South Macedonia and Thessaly, they had similar husbandry practices and they were keeping reliable reproductive records. All farms enrolled in the serological studies were free of brucellosis, they had conducted a BVD-MD eradication program, they were routinely applying vaccination program against BVD-MD, IBR, BSRV and PI3 and the reproduction

management was implemented in co-operation with staff members of our Clinic.

For the determination of serum antibody titres, 526 blood samples (102, 100, 116, 100 and 108 from negative and categories 1,2,3 and 4, respectively) were collected from the tail vein in plain vacutainers; blood was allowed to clot and serum was separated and stored at -20°C until it was assayed as it is described above, using an initial dilution 1/400.

To assess possible effects of infection on reproductive performance, comparisons were made 12 months after the initial laboratory detection of antibody titres.

Based on farm records, reproductive indices of individual cows were calculated. To compare possible effects of *C. burnetii* infection on reproductive parameters pairs of seronegative – seropositive cows were made in a category 3 farm, using as a criterion the calving date.

Pregnancy diagnosis was performed 35-42 days after AI and it was confirmed 60 to 75 days later. Abortion was defined any premature expulsion of a dead fetus or the failure of a cow to retain the pregnant status in both pregnancy examinations. As extended cycles were defined the interestrus intervals that exceeded 25 days, but they were not the product of multiplication by 20 to 24.

Statistical analysis

The number of AIs/pregnancy and calving interval among groups were compared by ANOVA followed by Duncan's new multiple range test. The results are expressed as mean \pm SEM. Among groups, chisquare analysis was used to compare the proportion of abortions, the proportion of cows with enlarged estrous cycle length, with retained fetal membranes or with uterus infection. Statistical differences were considered significant when $P < 0.05$.

Paired data within the same herd were individually analyzed by student's t-test.

Results

Twenty eight out of the 80 tested herds had antibodies in the bulk milk sample (prevalence 35%). According to the antibody titre, 3, 4, 16 and 5 herds (3.25%, 5%, 20% and 6.25%) were allotted to the categories 1, 2, 3 and 4, respectively.

Table 1. Distribution of seropositive animals in farms of all 5 categories.

Group	Herd Prevalence (positive/total number tested)	Prevalence of category 3-4 on total (number of animals/total)	Prevalence of category 3-4 on total seropositive
0	4,90 ^c (5/102)	0 ^d (0/102)	0 ^c (0/5)
1	25,00 ^b (25/100)	4,00 ^c (4/100)	16,00 ^b (4/25)
2	15,52 ^b (18/116)	5,17 ^c (6/116)	33,33 ^b (6/18)
3	24,00 ^b (24/100)	15,00 ^b (15/100)	62,50 ^a (15/24)
4	46,30 ^a (50/108)	35,16 ^a (38/108)	76,00 ^a (38/50)

^{a,b,c} Values with different superscript in the same column differ significantly (P<0,05)

Table 1 shows the distribution of animals with different seropositivity in each farm category. Seropositive animals of category 1 were detected in the farms that according to the bulk milk analysis were initially characterized as being negative.

Among groups, no differences were detected in the required number of AIs per pregnancy. Significant variations were detected in calving interval that was the highest in category 3 herds (table 2). No differences were detected in the proportion of abortions, while significant variations were found in proportion of: non-physiologically extended estrous cycle length, animals with retained fetal membranes and uterine infections (table3).

When reproductive indices of seropositive and seronegative cows in the same farm were studied, no statistical difference was detected between the two groups and, hence, the data are not presented in detail.

Table 2. Relationship of antibody titre in bulk milk sample and number of AIs per pregnancy and calving interval in positive and negative herds.

Category	AI (mean±SEM)	Calving interval (mean±SEM)
0	2,52±0,20 ^a	432,12±6,85 ^c
1	2,97±0,25 ^a	467,81±10,89 ^{a,b}
2	2,65±0,26 ^a	445,10±10,98 ^{b,c}
3	2,71±0,25 ^a	473,87±11,43 ^a
4	2,34±0,22 ^a	424,61±7,99 ^c

^{a,b,c} Values with different superscript in the same column differ significantly (P<0,01-0,001)

Table 4 shows data on reproductive indices from the paired seropositive and seronegative from a representative category 3 herd. Only the age of the cows tended (p=0,116) to differ between the two groups.

Table 3. Relationship of antibody titre in bulk milk sample and the rates of abortion, estrous cycle length, retained fetal membranes, and uterine infections in herds categorized according to bulk milk antibody titre.

Category	Abortion rate	Extended cycles rate	Retained Fetal Membrane rate	Uterine infection rate
0	5,42	16,83 ^b	11,79 ^{a,b}	16,51 ^b
1	6,02	12,10 ^c	9,04 ^b	13,86 ^b
2	3,89	22,66 ^a	15,56 ^a	24,12 ^a
3	6,15	14,11 ^{b,c}	15,16 ^a	20,90 ^{a,b}
4	6,75	18,52 ^a	10,91 ^{a,b}	15,06 ^b

^{a,b,c} Values with different superscript in the same column differ significantly (P<0,05)

Table 4. Reproductive indices of seropositive and seronegative animals from a category 3 herd..

	Number of animals	Age (months)	AI interval	AIs/ pregnancy	Calving interval	Mean numbers of calvings
Negative	11	40,9±14,5	28,7±18,9	2,9±1,6	441,0±49,5	1,8±1,2
Positive	11	48,9±11,3	35,6±22,9	2,6±1,2	420,3±32,5	2,4±0,8

Discussion

Here, we report for the first time the prevalence of *C. burnetii* infection in Greek dairy cattle farms. We provide evidence that antibody positive herds are prevalent in Greece; however, the disease had been underestimated so far. The prevalence of infection was 35%, which falls within the range reported in the international literature (Agger et al. 2010, Kim et al. 2005, Kim et al. 2006, Paiba et al. 1999, Lange et al. 1992, Lang 1988).

The sensitivity and specificity of the ELISA assay used in the present study are unknown and, hence, the true prevalence of the disease may be different from what is reported herein. Nonetheless, most research groups utilize either ELISA or complement fixation test for bulk milk antibody detection, which appear to be similar in terms of sensitivity and specificity (Hansen et al. 2011, Schalch et al. 1998). Seropositive animals were found in herds that were characterized as negative on the basis of antibody presence in the bulk milk. In these herds, the prevalence and the titre of seropositive animals were low, indicating that animals with low serum antibody titre excrete minimal antibody concentration in milk, which being diluted in the milk tank, further lowered milk titre to the negative zone of the assay. Similar findings have been reported by others (Hansen et al. 2011).

According to the answers we received during the farmers' interview (data not shown), no diagnosed human cases were reported in the personnel of farms enrolled in this study. This is either because, in fact, no infections have occurred or people had been infected, but the disease was mild and, hence, no medical consultancy was required.

No associations were found in prevalence between herds having importing replacing animals and those using exclusively own replacement heifers. This is possibly because the bacterium is highly contagious and can be carried for long distances by the dust and winds. In addition, infection could have occurred from small ruminants that graze in short distances in almost all farms.

In the present study, it appears that the prevalence of *C. burnetii* titres is not associated with reproductive disturbances. In fact, in the statistical analysis between farms, there were significant differences, which, nonetheless, could not be biologically interpreted under the

light of antibody titre. For example, in negative and grade 4 farms, incidence of uterine infection was not different, but in both groups it was lower than that of grade 2 farms. Similarly, negative and grade 4 farms had similar calving intervals that were lower from that of grade 3 farms. On the other hand, no differences were detected on the number of AIs per pregnancy or in the abortion rates. These -for the first view- paradox findings suggest that the differences reflect either different management practices or the existence of obscure pathological conditions between farms that were not evaluated in the present study or, finally, that the disease is asymptomatic in cattle. The latter hypothesis is a matter of controversy. Some researchers have reported that *C. burnetii* infection can cause or is associated with abortion in cattle (Bidfell et al. 2000, Arricau-Bouvery et al. 2006, Cabassi et al. 2006). In the latter study, antibody against *C. burnetii* were detected both in aborted cows (44.9%) and in cows that carried pregnancy to term (22%). Others have reported that the infection in cattle is subclinical and rather asymptomatic (Hansen et al. 2011, Rodolakis et al. 2007, Paiba et al. 1999, Behymer et al. 1976). The pathophysiology of abortion includes either the death of the fetus and/or placental lesions caused by placentitis as a result of colonization by the infectious agent. In goats, after experimental infection, *C. burnetii* was detected by PCR in all placentas and in several organs from the aborted fetuses (Arricau-Bouvery et al. 2003), while, in a field study, the microorganism was detected in the 20-25% of the sampled material from aborted ruminants indicating that the microorganism was the causative factor of the abortion (Jones et al. 2010). However, in the latter study, the prevalence of fetal infection was very high in goat fetuses and non-existing in the cattle; despite the small number of cases, this study may reveal that the susceptibility to the infection in ruminants is species-related. In addition, in a very recent study, it has been demonstrated that almost no placenta inflammation was detected in parturient cows originating from *C. burnetii* infected herds, which is a strong evidence, that in the bovine, the disease is mainly subclinical and asymptomatic (Hansen et al. 2011, Rodolakis 2009). This is confirmed in the present study, since no difference in abortion rate was found and, most importantly, no association could be made in reproductive indices between seropositive and seronegative herdsmates.

The present study revealed for the first time the presence of *C. burnetii* in dairy cows throughout central and Northern Greece. Though the present study does not elucidate associations between antibody titres and reproductive failures, the zoonotic nature of the disease dictates the need of a large national surveillance to be conducted.

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Conflict of interest

All authors declare that have no relationship with people or organizations that could prejudice or bias the content of this paper.

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