

Excretion of *Coxiella burnetii* in Cows with Secretion Disorder

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Abstract

Coxiella burnetii is the causative agent of a very important disease with zoonotic potential. Domestic ruminants are very important factor in transmission of this pathogen to humans. This pathogen can be found in feces, urine, lochia, placenta and milk of infected animals. Of all these routes, the excretion through milk lasts the longest, and can last over several lactations. In this paper, we examined the excretion dynamics of *Coxiella burnetii* through milk of seropositive cows and the levels of immunoglobulin G in milk and blood serum. Correlation between these parameters is also examined. Milk samples from infected cows were used for somatic cell count determination. The PCR method was used for determination of *Coxiella burnetii* genome. RID plates were used for determination of concentration of immunoglobulin. The excretion of the pathogen through milk differed between different stages of lactation: it was the lowest in the first stage (16.6%) and the highest in the second stage (80.0%). In the second stage the lowest concentration of immunoglobulin G was detected in milk serum (4.0 g). Somatic cell count was increased, some samples had very high values.

Keywords: *Coxiella burnetii*, Cow, Immunoglobulin, Somatic cells

Salgı Bozukluğu Olan İneklerde *Coxiella burnetii*'nin Atılımı

Özet

Coxiella burnetii zoonotik potansiyele sahip çok önemli bir hastalığa neden olan bir ajandır. Etkeni insanlara aktarmada evcil ruminantlar oldukça önemlidir. Etken enfekte hayvanların dışkı, idrar, vajinal akıntılar, plasenta ve sütlerinde bulunabilir. Tüm bu yollar arasında süt ile salınım en uzun süren salınım yolu olup birkaç laktasyon periyodu süresince devam edebilir. Bu makalede, seropozitif ineklerde süt yoluyla *Coxiella burnetii* atılımı ile süt ve kan serumunda immunoglobulin G seviyelerinin dinamikleri incelendi. Ayrıca bu parametreler arasındaki ilişki de değerlendirildi. Enfekte ineklerden elde edilen süt örneklerinde somatik hücre sayımı yapıldı. *Coxiella burnetii* genomunu belirlemek amacıyla PCR metodu uygulandı. İmmünoglobulin konsantrasyonunu belirlemek için RID plaklar kullanıldı. Patojenin süt ile salınımı laktasyon devreleri arasında farklılık gösterdi; birinci evrede en düşük olup (%80.0) ikinci evrede en yüksek seviyedeydi (%16.6). Süt serumunda immunoglobulin G konsantrasyonu en düşük ikinci evrede belirlendi (4.0 g). Somatik hücre sayısı artmış olup bazı örneklerde çok yüksekti.

Anahtar sözcükler: *Coxiella burnetii*, İnek, İmmunoglobulin, Somatik hücre

INTRODUCTION

Q fever is a zoonosis caused by an obligate intracellular microorganism, *Coxiella burnetii*. The disease is present throughout the world. The most important source for human infection is marked to be domestic animals. Dogs and cats are responsible for spread of disease primarily in urban areas. Ruminants are known to be the most important source of infection in humans [1,2]. In domestic animals, Q fever mostly passes as a latent disease and the commonest clinical symptoms are abortions and reduced fertility. In addition to these symptoms related to the reproductive

tract, occurrence of pneumonia, mastitis and polyarthritis have also been observed [2]. The causative agent of Q fever - *Coxiella burnetii* is an immobile Gram negative bacterium, its life cycle is completed in phagosomes of infected cells [3]. It has a cell membrane similar to that of other Gram-negative bacteria. It is normally stained with Giemsa since they stain poorly with the Gram stain [4]. *Coxiella burnetii* is very virulent, so the infectious dose could be only one microorganism [5].

The pathogenesis of this disease is characterized with primary replication in the lymph nodes, followed



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by stage of bacteremia and after that, localization of agent in predilection organs: primarily in mammary gland and uterus in pregnant animals [6]. Localization of pathogens in the mammary gland is critical for long-term secretion through milk, so the cows can excrete the agents through milk more than a year and even during successive lactations [7], while secretion through feces and vaginal discharge lasts for a few weeks. Reservoirs of the agent are only partially known, but certainly include mammals, birds and arthropods, especially ticks. Although 40 species of ticks can be naturally infected with *Coxiella burnetii*, they obviously do not have a great importance for the infection of animals and humans [8]. However, the pathogen replicates in cells of the tick's gut and it is excreted in large number through the feces. Leather and wool contaminated with feces of ticks can be a source of infection either through direct contact or after inhalation of dry feces. The farm animals - cattle, sheep and goats - are the most common source for human infections. Pets like dogs, cats or rabbits can also be source of infection with *Coxiella burnetii*. There is a report on the occurrence of disease in humans as a result of direct or indirect contact with cats during parturition [9]. In the case of dairy cows, occupational exposure of humans to infection should be highlight. Most exposed are veterinarians, farmers, milkmen and workers in slaughterhouses and dairies. In the general population, categories at risk are smokers and immunocompromised persons [10]. Some authors [11] have also reported a significant association between seropositivity in humans and intake of non-pasteurized milk and milk products, whether people were in contact with animals or not.

While *Coxiella burnetii* is shed for extended periods in milk of dairy cattle, and has been shown to be immunogenic in dairy cattle, potential associations with clinical or subclinical mastitis only rarely have been examined [12]. A more recent report suggested that the prevalence of *Coxiella burnetii* infections was higher among dairy cattle with reproductive problems including mastitis [13]. For detection of *Coxiella burnetii* PCR method is highly sensitive and specific detection method that has been used previously to trace *Coxiella burnetii* in clinical samples. A PCR performed with primers based on a repetitive, transposonlike element (Trans-PCR) proved to be highly specific and sensitive, but extraction of DNA from milk samples took considerable effort and there was a high risk of contamination due to the numerous preparation steps [14]. The objective of this work was to explore the potential association between *Coxiella burnetii* detection in milk by PCR and elevated SCC in milk in successive lactation stages and also to recognize relation between immunoglobulin G in milk serum and PCR detection in milk. Findings of agent in milk does not always coincides with serological finding, so seronegative animals can also excrete *Coxiella burnetii* in milk [15].

MATERIAL and METHODS

Serological screening of blood serum samples for antibodies to *Coxiella burnetii* was performed on a farm with 200 holstein-friesian dairy cows by ELISA. Serum samples were tested for Q fever antibodies using the indirect ELISA kit (Idexx Switzerland, Switzerland), according to the protocol recommended by the manufacturer. Sera were prepared at 1:400 dilution, and specific antibodies were detected using a peroxidase-labeled anti-ruminant immunoglobulin G (IgG) conjugate. Results were expressed as a percentage of the optical density reading of the test sample (value), calculated as $\text{value} = 100 \times (S-N)/(P-N)$, where S, N, and P are the OD of the test sample, the negative control, and the positive control, respectively. Sera were considered to be ELISA positive if they had a value of 40% or more, suspect if the value was between 30% and 40%, and negative if the value was <30%.

Based on the results of the ELISA tests, an experimental group of cows serologically positive for *Coxiella burnetii* was formed. In total, the experiment included nine dairy cows. The cows were in good body condition and showed no clinical signs of disease. In beginning of experiment all cows were in first lactation, and three of them newer got pregnant again. From another six, who got pregnant, one had abortion and five had normal calving.

From the experimental animals, milk samples during lactation, pregnancy and the postpartum period were collected during regular milking. With use of true-testers, from each cow two milk samples were taken. One sample was used for performing PCR and for determining of immunoglobulin G concentration in milk serum. Another sample was used for determination of somatic cell count.

In total 65 milk samples were taken during different stages of lactation. Along with milk samples, blood samples were also taken for determination of presence of *Coxiella burnetii* in serum.

After arriving in the laboratory, milk samples were placed in an incubator for 24-48 h. Incubation is carried out at a temperature of 38°C to form coagulum and milk serum. Blood samples were stored in room temperature for 48 h to segregate the serum. The PCR method was used to determine the presence of *Coxiella burnetii* genome in milk and blood serum samples. For serum samples, a 200- μ l sample volume was used. Cells were lysed with proteinase K (final concentration, 200 μ g/ml) at 56°C overnight. DNA was prepared with a Prep-A-Gene purification kit (Bio-Rad, Munich, Germany) by using 10 μ l of silica matrix. DNA was eluted from the silica matrix by adding 100 μ l of Prep-A-Gene elution buffer. To increase the yield, DNA was eluted at 56°C for 5 min and centrifuged again. One microliter of supernatant containing DNA was used for amplification. Followed

primers were used: Trans1: 5'-TGGTATTCTTGCCGATGAC-3';
Trans 2: 5'-GATCGTAACTGCTTAAT AAACCG-3'.

To determine the concentration of immunoglobulin G in milk and blood serum immunodiffusion method with RID plates was used. The RID plates with monospecific antiserum to bovine immunoglobulin G were provided by INEP (Belgrade, Serbia). Sample of milk serum was poured in wells of RID plate, and after incubation for 48 h in room temperature reading of results was done. Reading was done by measuring the diameter of the precipitation ring. Diameter was measured by RID meter with an accuracy of 0.1 mm. The value obtained using the following formula calculates the concentration of immunoglobulin in the tested serum.

The formula for the calculation is: $C = ((R^2 - b)/a) \times 30$, where R is the radius of precipitation ring, b is a constant whose value is 8.69, a is a constant with a value of 47.48. The resulting value is the concentration of immunoglobulin in the serum.

The correlation coefficient between IgG concentration and presence of *Coxiella burnetii* in milk serum was calculated using Statistica v. 7.5 software.

Research is approved by Ethics Commission to safeguard the welfare of experimental animals of the University of Novi Sad, number 01-153/7-3.

RESULTS

Processing of blood serum samples from 200 cows on tested farm by ELISA test has shown antibodies for *Coxiella*

burnetii in 9 cows. These animals accounted for 4.5% of total herd.

From seropositive cows, 65 samples of milk serum were collected by successive lactation stages. The results of the analysis of these samples using the PCR method are shown in Table 1. During lactation, the excretion of bacteria was greatest in the second stage when 80% of milk serum samples were positive for *Coxiella burnetii*. In the colostrums stage, there was a high percentage of *Coxiella burnetii* excretion through milk (50% of positive milk serum samples). The lowest percentage of excretion through milk was in the first stage of lactation (Table 1).

In Table 2 presence of *Coxiella burnetii* in blood serum of infected cows is shown. From Table 2 it can be seen that during all lactation stages there was a small oscillation in presence of agent in blood serum.

Concentration of immunoglobulin G in milk serum of infected cows is shown in Table 3. Highest concentration was in colostrums stage and significantly lower concentration was measured in successive lactation stages.

Somatic cell count in cumulative milk samples was measured during lactation stages and results are shown in Table 4. It is evident that SCC in milk samples from infected cows was increased during all lactation stages and some samples had very high values.

The correlation coefficient between presence of *Coxiella burnetii* genome in blood serum and excretion in colostrums and milk during all lactation stages was 0.072.

The correlation coefficient between excretion of *Coxiella*

Table 1. Excretion of *Coxiella burnetii* through milk in different lactation stages

Tablo 1. Farklı laktasyon evrelerinde süt yoluyla *Coxiella burnetii* atılımı

Stage of Lactation	Colostrals Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	4	8	20	33
<i>C. burnetii</i> excretion	50%	16.6%	80%	40.6%

Table 2. Presence of *Coxiella burnetii* in blood serum in different lactation stages

Tablo 2. Farklı laktasyon evrelerinde kan serumunda *Coxiella burnetii* varlığı

Stage of Lactation	Colostrals Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	8	21	30	6
<i>C. burnetii</i> in blood serum	50 %	66.6%	66.6%	48.6%

Table 3. Concentration of immunoglobulin G in milk serum in different lactation stages

Tablo 3. Farklı laktasyon evrelerinde süt serum immünoglobülin G konsantrasyonu

Stage of Lactation	Colostrals Stage First 10 Days	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
Number of samples	4	8	20	33
IgG concentration g/L	153.1±31.2	5.0±0.9	4.0±2.2	8.4±5.4

Table 4. Somatic cell count in cumulative milk samples from infected cows**Tablo 4.** Enfekte ineklerin kümülatif süt örneklerinde somatik hücre sayısı

Lactation Stage	First Stage 10-60 Days	Second Stage 60-180 Days	Third Stage Over 180 Days
SCC x 10 ³ /mL	147	526	1024
	778	1795	103
	2277	900	2000
	1004	588	1193
	419	169	836
	1092	685	236
	113	743	1236
	361	1485	1048
	-	1240	981
Average	773±711	903±512	961±558

burnetii through milk and of immunoglobulin G concentration in milk serum at each stage of lactation was 0.072.

DISCUSSION

Q fever disease caused by *Coxiella burnetii*, is an important zoonosis found worldwide. In humans, it causes a variety of diseases such as acute flu-like illness, pneumonia, hepatitis, and chronic endocarditis. In animals, *Coxiella burnetii* is found in the reproductive system, both uterus and mammary glands, and may cause abortion or infertility [16].

The high prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive problems showed that these infected cattle play an important role in maintaining the infection and in disseminating the pathogenic agent to environment. Thus, such excretions (milk, colostrums, urine, and birth fluid) are considered to be potential sources of infection in animals and humans via inhalation of infectious aerosols or airborne dust [13].

According to our results (Table 1), the lowest percentage of excretion of *Coxiella burnetii* was in the first stage of lactation, amounting to 16.6%. With the transition to the next stage of lactation, a striking increase in the percentage of excretion was noticed. In the second stage it was 80%. In the third stage there was decrease to 40.6%. In colostrums stage percentage of excretion through milk was 50%. Similar results are published by Rodolakis et al. [17] who claim that excretion of *Coxiella burnetii* through milk starts after eight to twelve weeks of lactation in most cows. This period coincides with second stage of lactation as we divide it.

In blood serum of infected cows presence of *Coxiella burnetii* was similar during all four lactation stages ranging from 48.6% to 66.6%. Presence of *Coxiella burnetii* genome in blood serum is consequence of lysis of infected cells

by antibody-dependent system. So bacteria became free in blood serum [18]. Reflecting these percents to whole herd it can be calculated that 2.4% to 3.3% of all animals have positive blood serum to *Coxiella burnetii*. This is similar to findings of Kirkan et al. [19]. Concentration of immunoglobulin G in milk serum was highest in colostrums stage (Table 3), this is in accordance with Leyton et al. [20]. Through the next two stages there was significantly lower concentration of immunoglobulin so the lowest was in second lactation stage (4.0 g/L). This is also the stage with highest excretion of *Coxiella burnetii* through milk. In third lactation stage there was increased immunoglobulin concentration. These values are in accordance with allegations of Bobos [21].

Analyzing values of somatic cell count in milk samples from infected cows it can be concluded that during whole lactation infected cows had increased number of somatic cells, with some samples having very high values. Barlow et al. [12] and Radinović et al. [22] had similar finding examining the milk from cows with *Coxiella burnetii* infection.

Infected cows shed *Coxiella burnetii* through milk during whole lactation with highest intensity in the second stage, while presence of pathogen in blood serum is similar in all lactation stages. Concentration of immunoglobulin G in milk serum corresponds to values in uninfected cows. Somatic cell count is increased in infected cows during all lactation stages.

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REFERENCES

- Arricau-Bouvery N, Rodolakis A:** Is Q fever an emerging or re-emerging zoonosis. *Vet Res*, 36 (3): 327-349, 2005. DOI: 10.1051/vetres:2005010
- Vidić B, Boboš S, Savić S, Prica N:** Findings of *Coxiella burnetii* in milk and her significance for human infection. *Contemp Agri*, 57 (3-4): 208-215, 2008.
- Woldehiwet Z:** Q fever (coxiellosis): epidemiology and pathogenesis. *Res Vet Sci*, 77 (2): 93-100, 2004. DOI: 10.1016/j.rvsc.2003.09.001
- Gimenez DF:** Staining rickettsiae in yolk-sack cultures. *Stain Technol*, 39 (3): 135-140, 1964.
- McQuiston JH, Childs JE, Thompson HA:** Q fever. *J Am Vet Med Assoc*, 221 (6): 796-799, 2002.
- Babudieri B:** Q fever: A zoonosis. *Adv Vet Sci*, 5, 82-182, 1959.
- Muskens J, van Engelen E, van Maanen C, Bartels C, Lam TJ:** Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet Rec*, 168 (3): 79, 2011. DOI: 10.1136/vr.c6106
- Maurin M, Raoult D:** Q fever. *Clin Microbiol Rev*, 12 (4): 518-553, 1999.
- Marrie TJ, Raoult D:** Update on Q fever, including Q fever endocarditis. *Curr Clin Top Infect Dis*, 22, 97-124, 2002.

- 10. Van Der Hoek W, Dijkstra F, Schimmer B, Schneeberger PM, Vellena P, Wijkmans C, ter Schegget R, Hackert V, van Duynhoven Y:** Q fever in the Netherlands: An update on the epidemiology and control measures. *Euro Surveill*, 15 (12), pii: 19520, 2010.
- 11. Cerf O, Condron R:** *Coxiella burnetii* and milk pasteurization: An early application of the precautionary principle? *Epidemiol Infect*, 134 (5): 946-951, 2006. DOI: 10.1017/S0950268806005978
- 12. Barlow J, Rauch B, Welcome F, Kim SG, Dubovi E, Schukken Y:** Association between *Coxiella burnetii* shedding in milk and sub-clinical mastitis in dairy cattle. *Vet Res*, 39, 23, 2008. DOI: 10.1051/vetres:2007060
- 13. To H, Htwe KK, Kako N, Kim HJ, Yamaguchi T, Fukushi H, Hirai K:** Prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive disorders. *J Vet Med Sci*, 60 (7): 859-861, 1998. DOI: 10.1292/jvms.60.859
- 14. Lorenz H, Jager C, Willems H, Baljer G:** PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. *Appl Environ Microbiol*, 64, 4234-4237, 1998.
- 15. Rousset E, Berri M, Durand B, Dufour P, Prigent M, Delcroix T, Touratier A, Rodolakis A:** *Coxiella burnetii* shedding routes and antibody response after outbreaks of Q fever-induced abortion in dairy goat herds. *Appl Environ Microbiol*, 75 (2): 428-433, 2009. DOI: 10.1128/AEM.00690-08
- 16. Porter S, R, Czaplicki G, Mainil J, Guatteo R, Saegerman C:** Q fever: Current state of knowledge and perspectives of research of a neglected zoonosis. *Int J Microbiol*, 2011. DOI: 10.1155/2011/248418
- 17. Rodolakis A, Berri M, Héchard C, Caudron C, Souriau A, Bodier CC, Blanchard B, Camuset P, Devillechaise P, Natorp JC, Vadet JP, Arricau-Bouvery N:** Comparison of *Coxiella burnetii* shedding in milk of dairy bovine, caprine, and ovine herds. *J Dairy Sci*, 90 (12): 5352-5360, 2007. DOI: 10.3168/jds.2006-815
- 18. Jerrells TR:** Mechanisms of immunity to Rickettsia species and *Coxiella burnetii*. In, Walker DH (Ed): *The Biology of the Rickettsiae*. Vol. II. 79-100, CRC Press, Boca Raton, FL, 1988.
- 19. Kirkan S, Kaya O, Tekbiyik S, Parin U:** Detection of *Coxiella burnetii* in cattle by PCR. *Turk J Vet Anim Sci*, 32 (3): 215-220, 2008.
- 20. Leyton W, Gapper, David EJ, Copestake, Don E, Otter, Indyk Harvey E:** Analysis of bovine immunoglobulin G in milk, colostrums and dietary supplements: A review. *Anal Bioanal Chem*, 389 (1): 93-109, 2007. DOI: 10.1007/s00216-007-1391-z
- 21. Boboš S, Vidić B:** Mlečna žlezda preživara- morfologija- patologija- terapija. Novi Sad: Poljoprivredni fakultet- Departman za veterinarsku medicinu, Naučni institut za veterinarstvo „Novi Sad“; 2005.
- 22. Radinović M, Boboš S, Pajić M, Vidić B, Rašić Z, Davidov I, Špegar R:** Blood count and number of somatic cells in milk of cows infected with *Coxiella burnetii*. *Vet Glasnik*, 65 (5-6): 367-374, 2011. DOI: 10.2298/VETGL1106367R