

Investigation of Q Fever in Kilis and Shamil goats by ELISA and Touchdown-PCR

Elçin GÜNAYDIN¹, Selçuk PEKKAYA², H. Kaan MÜŞTAK³, Bestami DALKILIÇ⁴

¹ Veterinary Control Central Research Institute, Breeding Disease Laboratory, Etlik, Ankara; ² Veterinary Control Central Research Institute, Biochemistry Laboratory, Etlik, Ankara; ³ Ankara University Faculty of Veterinary Medicine, Department of Microbiology, Dışkapı, Ankara; ⁴ University of Gaziantep, Vocational School of Higher Education, Gaziantep, Turkey.

Summary: This study was carried out on Kilis and Shamil goats to determine the different stages of Q fever-chronic and acute- in serum samples by indirect ELISA and in blood samples by touchdown-PCR, respectively. A total of 92 serum samples comprising 46 Kilis and 46 Shamil goats was examined and 7.60%, 4.34% and 10.8% seropositivity were determined for total serum samples, Kilis and Shamil goat serum samples, respectively. Of the examined 92 blood samples belonging to 46 Kilis and 46 Shamil goats, none of them yielded 687 bp PCR products by touchdown-PCR. The positive ELISA titers were thought to be the evidence of previous infection. The province of Kilis localized in South-East Anatolian Region of Turkey was decided to be a risky region for Q fever.

Key words: Q fever, goat, ELISA, touchdown-PCR

Kilis ve Halep keçilerinde ELISA ve Touchdown-PCR ile Q Humması'nın araştırılması

Özet: Bu çalışma, Kilis ve Halep keçilerinde, Q Humması'nın farklı dönemlerini -kronik ve akut- sırasıyla; serum örneklerinde indirek ELISA, kan örneklerinde touchdown-PCR ile ortaya koymak için planlanmıştır. Kırkaltı Kilis ve 46 Halep keçisinden oluşan toplam 92 serum örneği incelendi ve toplam serum örnekleri, Kilis ve Halep keçi serumlarında seropozitiflik sırasıyla, 7.60%, 4.34% ve 10.8% bulundu. Kırkaltı Kilis ve 46 Halep keçisine ait toplam 92 kan örneğinin, hiçbirinde touchdown-PCR ile 687 bp'lık PCR ürünü tespit edilmedi. Pozitif ELISA titrelerinin daha önce geçirilen enfeksiyona kanıt olduğu düşünüldü. Türkiye'de Güneydoğu Anadolu Bölgesi'nde lokalize olan Kilis ilinin Q fever yönünden riskli bir bölge olduğuna karar verildi.

Anahtar sözcükler: Q humması, keçi, ELISA, touchdown-PCR

Introduction

Coxiella burnetii, an obligate intracellular bacterium, expressing phase variation related to the changes in lipopolysaccharide (LPS) is the causative agent of a zoonotic Q fever. Q fever is endemic throughout the world, occurring in diverse geographic regions and climatic zones (38). Ruminants, especially goat and sheep are the main reservoirs of Q fever and females may shed particles via vaginal secretions, faeces urine, and milk (2, 5, 6, 7).

C. burnetii infection is still considered to be a common cause of caprine abortion in several countries. Susceptible pregnant females develop necrotizing placentitis which results in abortion, whereas non-pregnant females do not develop clinical signs. After the initial abortions or infections, animals become immune to abortion but can remain subclinically infected. After the infection, the female can carry the organism indefinitely and sporadically shedding it with milk and at parturition (9). While acute Q fever infection occurs, antibodies against phase II antigens are observed in higher rates. In chronic infections,

antibodies against both phase I and phase II antigens are found to be in higher rates because of the fact that *C. burnetii* is a phase variable bacterium (26).

Investigations on Q fever mostly depend on serological examinations such as immunofluorescence, complement fixation test (CFT) and ELISA (28). The ELISA detects anti-phase I and anti-phase II antibodies whereas IFA detects only anti-phase II antibodies. Rousset et al. (28), declared that when the serum samples were measured for both IgM and IgG antibodies against either phase I and phase II antigens. Anti-phase II antibodies predominated after the primary infections and persist throughout the course of the infection, whereas higher titers of antibodies to the phase I antigen present only the chronic form of the illness. CFT, on the contrary to ELISA, is in capable of detecting all IgG subclasses. In ruminants, only IgG1 fixes the complement and can thus be detected by CFT. Moreover, IgG2, IgM and anticomplement substances potentially present in serum are capable of interfering with fixation of IgG1 to the complement, lowering the titer of IgG1 detected by CFT

(27). Rousset et al. (28), advised not to use CFT for serological animal screening because of its low sensitivity. Therefore, due to in capabilities of FAT and CFT mentioned above, ELISA is preferred in order to detect anti-phase I and phase II antibodies. However, seroconversion typically occurs 7–15 days after symptoms appear. For a definitive diagnosis in the early stages of acute Q fever, serologic testing in combination with PCR is recommended (34). Touchdown-PCR employs an initial annealing temperature above the projected melting temperature ($T(m)$) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles, consequently, the primers avoid amplifying nonspecific sequences and the specificity enhanced.

The purpose of this study was to investigate the different stages -chronic and acute- of the infection in female non-pregnant goats. For this purpose, serum and blood samples of Kilis and Shamil goats reared in the province of Kilis localized in South-East Anatolian Region of Turkey were examined by indirect ELISA and touchdown-PCR, respectively.

Material and Method

Serum and Blood Samples: In May 2012, total 92 serum samples (46 Kilis, 46 Shamil goats) and 92 blood samples (46 Kilis, 46 Shamil goats) were collected from the province of Kilis, Turkey. All the goats were 2-3 years old female, non-pregnant, selected randomly and none of them have a recorded abortion history or reproductive disorder. The samples were collected from each 8 different herds (Kilis and Shamil goats were collected ranging between 3 to 9 goats and 2 to 13 goats, from each herd respectively). Blood samples collected in blood tubes with EDTA anticoagulant for touchdown-PCR. All the sera for ELISA were separated 24 hours after sampling. All serum and blood samples were stored at -20°C in deepfreeze until tested.

ELISA: The serum samples were tested for the presence of antibodies directed to both phase I and phase II antigens of *C. burnetii* using ELISA CHECKIT Q-fever test (Idexx Laboratories, Broomfield, CO, USA) according to the manufacturer's instructions. Sera were prepared at 1:400 dilutions and specific antibodies were measured using a peroxidase-labeled anti-ruminant immunoglobulin G conjugate. Results were expressed as a percentage of the optical density reading of the test sample (OD %) calculated as $\text{S/P \%} = 100 \times (\text{S}-\text{N})/(\text{P}-\text{N})$ where S, N and P are the OD values of the test sample, the negative and positive controls, respectively. The OD of the positive control should not exceed 2.000 and OD of negative control should not exceed 0.500. The difference between the positive and negative control should be ≥ 0.300 . The serum samples were read at 450 nm as reported in the kit instructions. The interpretaion

of the results is as follows; $\text{S/P \%} < 30$ is negative; $\text{S/P \%} \geq 30$ and < 40 is suspected; $\text{S/P \%} \geq 40$ is positive.

Positive control in touchdown-PCR: DNA extracted from the phase II stage of *C. burnetii* in cell culture was kindly obtained from University of Firat, Faculty of Veterinary Medicine, Department of Microbiology and used as a positive control in touchdown-PCR.

The detection limit of touchdown-PCR with DNA extracted from phase II stage of *C. burnetii*: The initial concentration of DNA (undiluted DNA from phase II stage of *C. burnetii* obtained from cell culture) was measured as 5 ng/ μl by spectrophotometer (Nanodrop 1000). Ten fold dilution of the stock DNA was done from 10^{-1} to 10^{-5} (5 ng/ μl to 0.0005 ng/ μl). Each dilution was served as a template in touchdown-PCR.

DNA extraction: DNA was extracted from the blood samples by a commercial DNA isolation kit (QIAamp DNA Mini Kit; Qiagen, Cat no: 51104) according to the manufacturer's instructions. DNAs were stored at -20°C until used.

Primers: Trans-1 and trans-2 primers, complementary to the *IS1111* fragment, a transposon-like repetitive region were used for the diagnosis of *C. burnetii* by touchdown PCR. Primers as previously described by Hoover et al. (1992), (15) consisted of the following sequences: Trans 1; 5'-TAT GTA TCC ACC GTA GCC AGT C-3' and Trans-2; 5'-CCC AAC AAC ACC TCC TTA TTC-3'. Expected amplicon size was 687 bp.

Touchdown-PCR: Each reaction had a volume of 25 μl including, 22 μl reaction mixture consisting of 2.5 μl $10\times$ PCR buffer (without MgCl_2), 0.5 μl dNTP (10 mM), 1.5 μl MgCl_2 (25 mM), 1 μl of each primer (10 pmol/ μl), 0.25 μl Taq DNA polymerase (5 U/ μl) (Fermantas; EP 402), 15.25 μl deionized water and 3 μl template. Cycling parameters were as follows: initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation at 94°C for 30 sec, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, extension 72°C for 1 min and final extension 72°C for 10 min. Touchdown PCR was performed using Thermal Cycler (Arktik, ThermoScientific). PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer containing 0.5 $\mu\text{g/ml}$ of ethidium bromide at 100 V for 45 min and visualized under UV light.

Results

The sensitivity of touchdown-PCR: The detection limit of touchdown-PCR was determined as 0.005 ng/ μl (Fig 1).

ELISA results of field samples: Seven (7.60%) out of 92 serum samples were found to be antibody positive. Out of 46 serum samples belonging to Kilis goats, 2 (4.34%) antibody positivities were determined. Of the examined 46 Shamil goat serum samples, positive ELISA titers were determined in 5 (10.8%) serum samples. The

positive titers were mentioned in Table 1. S/P % were calculated and interpreted according to the kit instructions.

Touchdown-PCR results of field samples: Of the examined 92 blood samples comprising 46 Kilis and 46 Shamil goats, none of them yielded 687 bp amplification products by touchdown-PCR.

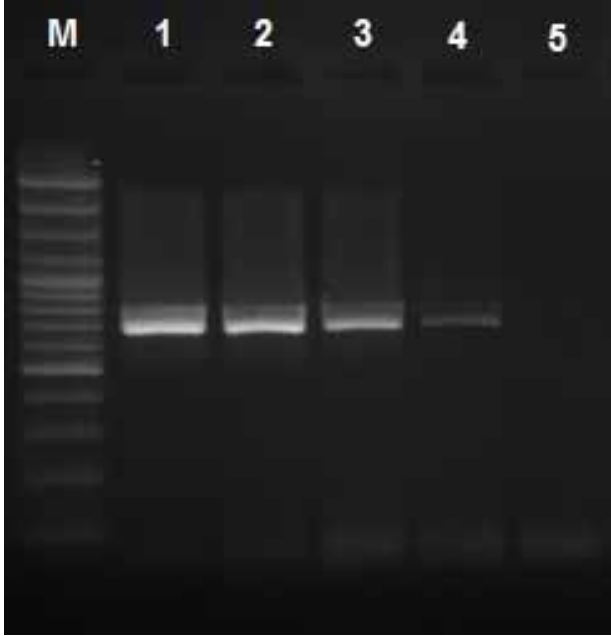


Figure 1. The sensitivity of touchdown-PCR.

M: 100 bp DNA marker (Fermentas; O'RangeRuler 100 bp DNA Ladder, # SM0623); Lane 1: 5 ng/μl (initial concentration of DNA), Lane 2: 5×10^{-1} ng/μl; Lane 3: 5×10^{-2} ng/μl; Lane 4: 5×10^{-3} ng/μl; Lane 5: 5×10^{-4} ng/μl

Şekil 1: Touchdown-PCR'in sensitivitesi.

M: 100 bp DNA marker (Fermentas; O'RangeRuler 100 bp DNA Ladder, # SM0623); Sıra 1: 5 ng/μl (başlangıç konsantrasyonu), Sıra 2: 5×10^{-1} ng/μl; Sıra 3: 5×10^{-2} ng/μl; Sıra 4: 5×10^{-3} ng/μl; Sıra 5: 5×10^{-4} ng/μl

Table 1. The positive ELISA titers (OD) and S/P % values.
Tablo1. Pozitif ELISA titreleri (OD) and S/P % değerleri.

Code of ELISA positive serum samples	Positive ELISA Titers (OD) at A_{450}	S/P % of positive serum samples
K*16	1.571	76.4
K33	1.170	54.4
S*5	1.203	56.2
S25	2.619	134.10
S28	1.142	52.86
S38	1.649	80.74
S46	1.161	53.90

Minimum titer (OD) at A_{450} : 0.046 and S/P % = -7.42 (Negative)

Maximum titer (OD) at A_{450} : 2.169 and S/P % = 134.10 (Positive)

K*: Kilis, S*: Shamil.

A_{450} dalga boyunda Minimum titre (OD) = 0.046 ve S/P % = -7.42 (Negatif)

A_{450} dalga boyunda Minimum titre (OD) = 2.169 ve S/P % = 134.10 (Pozitif)

K*: Kilis, H*: Halep.

Discussion and Conclusion

In the previous studies, ELISA results showing the herd level prevalence of *C. burnetii* infection in goat were ranged from 43% to almost 100% (14). Briefly, Q fever prevalence of goat herds determined by ELISA was 13% in Africa (31), 35% in Mexico (30), 5.7% in India (33), 65.8% in Iran (19). Individual prevalence of *C. burnetii* infection determined in goat by ELISA had been found to be between 5.7% and 75% (14). Since 2007 till now, Netherland has experienced human Q fever outbreaks associated with infected goat and sheep farms (1, 27, 35). A serological survey carried out in sheep and goats in Netherland in 2008, individually, 2.4% of the sheep and 7.8% of the goats were determined as antibody positive by ELISA (35). To our knowledge, a total of 10 regional seroprevalence studies conducted on cattle, sheep, goat in Turkey (3, 10, 13, 16, 18, 20, 22, 23, 32, 39). Of them all, solely in two studies, goat sera were examined for the presence of *C. burnetii* antibodies. Payzin et al. (1953) (23), determined 13% positivity of the examined 278 goat sera by CFT. Arserim et al. (2011) (3) reported that, 270 (38.6%) out of 700 goat sera were found to be positive by an ELISA.

In this study, we decided to determine the prevalence of Q fever in randomly selected, healthy, female, non-pregnant Kilis and Shamil goats, with no clinical signs. We had no knowledge about the tested animals such as any recorded abortion history. Antibody positivity, 7.60%, 4.34%, and 10.8% in total 92 goat serum samples, 46 Kilis and 46 Shamil goat serum samples, respectively showed that, those were challenged with *C. burnetii* at any stage of their life. Due to detection capacity of the ELISA kit consisting of phase I, phase II antigens from the Nine Mile Strain of *C. burnetii*, and peroxidase labeled anti-ruminant IgG conjugate, antibodies against both phase I and II could be easily detected, but not discriminated. As the sera were not tested by IFA, the positive antibody titers could not be attributed to an acute infection. However, persisting high level antibodies to phase I and phase II antigens, are thought to be caused by continuous antigenic stimulation and considered indicative for chronic Q fever in humans (37). In contrast to this, the importance of the development of antibody response against phase I and phase II antigens and the stage of the infection has not been well evaluated in animals. In this study, from our point of view, the positive titers cannot be attributed to onset of Q fever infection. However, the positive ELISA titers were lead us to think that those goats might have been infected with *C. burnetii* at any stage of their life as emphasized above. Serologic tests are not a useful tool to determine which animal represents a current risk for transmission, as animals may seroconvert without shedding or remain positive long after the acute infection. Reichel et al. (25), reported that, in six months

study, where sampling was scheduled 3 times with 2 months interval from aborted goats, positive antibody ELISA titers had been recorded on the second, even on the third sampling, suggesting a long duration of detectable antibody level. Conversely, some animals may pose a risk for infection prior to development of antibodies by shedding the bacteria and some infected animals never seroconvert (8). In the absence of longitudinal and repeating sampling, seroprevalence studies do not provide information on incidence of current infection in animals, or whether an animal is infectious (30). Hence, the positive ELISA titers were thought to be the evidence of previous infections. Similarly, EFSA stated that individual serology cannot be relied upon to confirm the presence of *C. burnetii* infection (12). Reichel et al. (25), declared that, positive ELISA results were observed in goats aborted and also in goats without any abortion history. The similar result was also reported by Rousset et al. (27).

When we tested the blood samples of the serologically examined goats for presence of *C. burnetii* DNA by a touchdown-PCR, none of them, gave the expected size of the amplicon, 687 bp, showing that the seven antibody positive goats were in the chronic stage of the infection. As previously stated by Musso et al. (21), blood sample was suitable for the peracute stage of the infection in order to detect *C. burnetii* DNA. After primary multiplication in the regional lymph nodes, ensuing bacteraemia lasts for 5-7 days and the organism then localizes in the mammary glands and the placenta of pregnant animals (4). ELISA results were overlapped PCR results, since seroconversion typically occurs 7–15 days after symptoms appear and due to detectable ELISA titers, it was expected not to determine *C. burnetii* DNAs in the blood samples.

All the tested goats are female. The prevalence rate of Q fever is found to be higher in females than in males since females are more susceptible to Q fever than males. In addition, hormonal changes during pregnancy results with the increased multiplication of *C. burnetii* in the placenta (24). Yet, we had no knowledge about goat's history. Anyway we could say, due to the gender of examined goats, those might have been prone to Q fever.

The geography, landscape and climate may also play a role in the spread of infection (36). The geographical features of Kilis is mountainous bordering to Syria and semi-mountainous in its vicinity with specific climatic conditions, varying from tropical-humid to hot-dry. The climate of Kilis can be termed as intersection point of Mediterranean and continental climates. Either geographical characteristics or climatic features of the province are appropriate for the dispersion of aerosols via air. The economy of Kilis depends on agriculture and animal husbandry. Prevalence of Q fever

in Shamil and Kilis goats were related to geographical variation, climatic conditions and economy of this region. Due to economic status in South-Eastern Anatolian Region of Turkey could lead inadequate animal husbandry conditions which might result in spreading various agents in the flocks, as previously stated by Cetinkaya et al. (10) and Arserim et al. (3).

Consequently, seropositivity in goats had a ratio that should not be ignored in the province of Kilis. Either geographical or climatic features of the province prone the animals to Q fever, therefore, veterinary authorities should take prompt preventive measures and raise awareness of animal breeders to take hygienic measures in order to restrict the probable infection.

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Address for correspondence:

Dr. Elçin Günaydın
 Veterinary Control Central Research Institute,
 Breeding Disease Laboratory,
 Etlik, Ankara, TURKEY.
 e-mail: elcin_gunaydin@hotmail.com