Identification of Chlamydophila abortus infection in aborting ewes and goats in Eastern Turkey

H. KALENDER1, A. KILIÇ2*, H. ERÖKSÜZ3, A. MUZ4, Ü. KILINÇ5, B. TAŞDEMİR6

1Süleyman Demirel Keban Vocational School, Firat University, 23119 Elazig, TURKEY;  2Sivrice Vocational High School, Firat University, 23119 Elazig, TURKEY;  3Department of Pathology, Faculty of Veterinary Medicine, Firat University, 23119 Elazig, TURKEY;  4Department of Microbiology, Faculty of Veterinary Medicine, Firat University, 23119 Elazig, TURKEY;  5Veterinary Control and Research Institute, 23119 Elazig, TURKEY.

*Corresponding author: akilic23@gmail.com

SUMMARY

This study was conducted to identify Chlamydophila abortus (C. abortus) by culture and PCR in aborting ewes and goats. During two successive lambing seasons, a total of 71 aborted foetuses (64 sheep and 7 goats, each from a different herd), and 100 vaginal swab samples from 10 of the herds in which abortions had been observed, have been analysed by culture and PCR. C. abortus was identified in 7 (6 lambs and 1 goat) of the 71 aborted foetuses (9.86%) using culture and PCR from embryonated egg yolks inoculated with foetal organ suspensions, and was detected directly from foetal organ suspensions in only 5 cases. The DNA was also identified in 6% of isolates in the vaginal swab samples as well as in the corresponding abortion products in 2 of the herds. All isolates showed identical restriction profiles with the S26/3 reference strain using AluI, HaeIII, and RFLP test. Focal necrosis was evidenced in foetal liver, spleen and lungs, as well as portal mononuclear cell infiltrates in the liver and alveolar septal thickening in the lungs. These findings show that C. abortus plays a substantial role in sheep abortion in Turkey.

Keywords: Chlamydophila abortus, ewe, goat, abortion, culture isolation, PCR, RFLP, Turkey

RESUME

Identification d’infections par Chlamydophila abortus lors d’avortements chez les brebis et les chèvres dans l’Est de la Turquie

Cette étude a été entreprise afin d’identifier Chlamydophila abortus (C. abortus) par culture et PCR lors d’avortements chez des brebis et des chèvres. Durant 2 saisons successives d’agnelage, 71 avortons (64 agneaux et 7 chevreux) provenant de troupeaux distincts chacun et 100 frottis vaginaux réalisées sur des femelles de 10 troupeaux différents dans lesquels des avortements ont été observés, ont été analysés par culture et PCR. C. abortus a été identifié chez 7 avortons (6 agneaux et 1 chevreux) et 71 recueillis (9.86%) par culture et PCR réalisée à partir de jaunes d’oeufs embryonnés inoculés par des suspensions d’organes fœtaux alors que la PCR réalisée directement à partir des suspensions d’organes fœtaux n’a été positive que dans 5 cas. L’ADN de C. abortus a aussi été mis en évidence dans 6% des frottis vaginaux ainsi que dans les avortons correspondants provenant de 2 troupeaux parmi les 10 étudiés. Tous les isolats ont présenté des profils de restriction identiques à celui de la souche de référence S26/3 en utilisant comme enzymes de restriction AluI et HaeIII lors de l’analyse RFLP (Restriction Fragment Length Polymorphism). Des foyers de nécrose ont été mis en évidence dans le foie, la rate et les poumons des fœtus avortés ainsi que des infiltrats de cellules mononucléées dans les espaces porte des foies et des épaissements des cloisons alvéolaires dans les poumons. L’ensemble de ces résultats montrent que C. abortus joue un rôle important dans les avortements des brebis en Turquie.

Mots-clés : Chlamydophila abortus, brebis, chèvre, avortement, isolation par culture, PCR, RFLP, Turquie.

Introduction

Chlamydiae are intracellular micro-organisms that cause different diseases in man and animals. According to a classification based on the phylogenetic analysis of their 16S and 23S rRNA genes, the family Chlamydiaceae consists of two genera, Chlamydia and Chlamydophila, and nine species [12].

Enzootic abortion of sheep, caused by Chlamydophila abortus (C. abortus), is an infectious disease characterized by placentitis and abortion; it has a negative effect on sheep breeding in many countries. C. abortus infection is also observed in goats and cattle [21, 29]. No clinical signs are present in the animals until abortion or delivery; animals who do not abort deliver very weak lambs [4]. The abortion generally occurs in the last 2-3 weeks of pregnancy. It has been reported that the abortion rate in affected herds is low in the first year, reaches or exceeds 30% in the second and is of the order of 5-10% in the third year [1]. Latent infections continuing longer than 3 years have also been reported [23].

Pathological changes in the course of C. abortus infection can be observed starting on the 90th day of pregnancy. Yellowish superficial exudate, red cotyledons and a red, thickened inter-cotyledon membrane are described as features of the placenta of sheep aborting in the 18th week [21]. Focal necrosis can be found in the liver of the foetus, as well as small focal areas of necrosis in the lungs, spleen, more rarely also in the brain and lymph nodes [8]. Histological examination of the placenta has shown vasculitis and thrombotic infiltrates of inflammatory cells in the cotyledonary membrane mesenchyme [7].
Direct microscopic examination, pathogen isolation, serological tests (complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and immunofluorescence), immunohistochemistry and DNA-based methods (polymerase chain reaction and DNA microarray) are used for diagnosis [2]. Serological testing is more frequently used in routine diagnosis because pathogen isolation is difficult and time-consuming. Cross-reactions between C. abortus and Gram-negative bacteria such as C. pecorum and Acinetobacter can be seen with CFT, which is recommended by The World Organization for Animal Health (OIE) [2, 31]. Recently, conventional and real-time PCR have been widely used to identify C. abortus in clinical samples. PCR methods for this are based on amplification of the chlamydial outer membrane protein genes ompA, omp1 and omp2, the polymorphic membrane gene pmp, genes encoding 16S rRNA and helicase, and the 16S-23S rRNA intergenic interval [5, 6, 14, 15, 22, 26, 32]. The use of rapid and reliable diagnostic tests is important in speedy disease control. The extent of C. abortus infections in Turkey has generally been documented serologically, while studies on pathogen isolation are relatively rare.

This study was performed to determine the extent of C. abortus infection in aborting ewes and goats in Eastern Turkey, and to compare pathogen culture and PCR methods for diagnosis.

**Material and Methods**

**SAMPLES**

The study was performed in 2008-2010, during two successive lambing seasons. A total of 71 aborted foetuses, 64 from sheep and 7 from goats, that each had been obtained from different herds and sent for diagnosis to the Elazığ Veterinary Control and Research Institute, and 100 vaginal swabs taken from 10 cows from the affected herds were used. The swabs were brought to the laboratory in SPG medium (Sucrose 0.218 mM, K2 HPO 4 7.1 mM, L-glutamic acid 4.9 mM, 10% foetal calf serum, streptomycin 100 µg/mL, nystatin 50 µg/mL, gentamicin 50 µg/mL).

**ISOLATION OF THE PATHOGEN**

A 10% suspension of foetal organ samples (liver, spleen and lung) was prepared in nutrient broth with streptomycin (200 µg/mL), 0.2 µL of which was inoculated into the yolk sac of 6-8 days-old embryonated chicken eggs, incubated 4-13 days at 37°C. The eggs which showed embryonic death were opened; preparations made from their yolk sacs were treated with the modified Ziehl-Neelsen (MZN) stain and studied for the presence of elementary corpuscles. Blind passages were made where no embryonic death had been observed [2, 33].

**RESULTS**

Of the material used as control during PCR, the C. abortus S26/3 strain was obtained from the Pendik Veterinary Control and Research Institute, the C. pecorum E58 and C. psittaci 6BC strains from Dr. Bernard Kaltenboeck (Auburn University, Alabama, USA).

The PCR products were digested with the AluI and HaeIII enzymes, using a mixture of 10 µL PCR product, 18 µL of nuclease-free distilled water, 2 µL 10X Buffer and 2 µL of the enzyme. The mixture was incubated overnight at 37°C, and then kept 20 minutes at 80°C or 65°C to inactivate HaeIII and AluI, respectively. The fragments were subjected to electrophoresis in 1.5% agarose gel at 70 V for 1 hour and examined in a UV transilluminator after 0.5 µg/mL ethidium bromide staining.

**HISTOLOGICAL EXAMINATION**

Tissue samples from the aborted foetuses (lung, liver, spleen) were fixed in buffered 10% formaldehyde. After inclusion in paraffin blocks, 4 µm sections cut in the microtome were stained by haematoxylin-eosin and examined under a light microscope.
CHLAMYDOPHILA ABORTUS INFECTION AND ABORTION IN EWES AND GOATS.

of the 100 vaginal swab samples (6.00%) obtained from ewes in herds with abortions (Table II). Direct samples from foetal tissues belonging to herds that were negative for vaginal swab examination were also negative.

After PCR amplification and agarose gel electrophoresis of the DNA extracted from yolk sac culture, vaginal swab samples and organ suspensions, a 479 bp band was observed from positive samples (figure 1). The primer pair used in the study was separately controlled against a C. pecorum E58 standard strain, which was found negative whereas the C. psittaci 6BC produced a positive band. Following digestion with the AluI and HaeIII enzymes, all isolates exhibited the same profile as the standard C. abortus S26/3 strain. No common fragment was found by analysis of the C. psittaci 6BC standard strain on RFLP (restriction fragments length polymorphism), differentiating it from the C. abortus isolates (figure 2).

It was interesting to note the relative integrity of the tissues of the examined foetuses. While the liver showed some swelling, blunting of the edges, and the presence of faint

<table>
<thead>
<tr>
<th>Herds</th>
<th>Number of vaginal swabs</th>
<th>Positive PCR: number (and %)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>3*</td>
<td>11</td>
<td>4 (36.36%)</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>6*</td>
<td>7</td>
<td>2 (28.57%)</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>0</td>
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<tr>
<td>9</td>
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<td>0</td>
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<tr>
<td>10</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
<td><strong>6 (6.00%)</strong></td>
</tr>
</tbody>
</table>

* samples from the aborted foetuses from these herds were PCR positive.

Table II: Detection of Chlamydia abortus by PCR from vaginal swab samples (n = 100).

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**Figure 1:** C. abortus PCR products; M: 100 bp DNA ladder, 1-6: Positive samples, 7: C. psittaci 6BC, 8: Negative control, 9: C. abortus S26/3.

**Figure 2:** RFLP (restriction fragments length polymorphism) analysis by AluI restriction enzyme of PCR products; M: 100 bp DNA ladder, 1: C. abortus S26/3, 2: Negative control, 3: C. psittaci 6BC, 4-10: Positive samples.
yellowish foci 1-2 cm in diameter on the cut surface, other organs did not have visible pathological changes. Generally subcapsular, focal or piecemeal areas of coagulative necrosis (figure 3) and lesions characterized by slight mononuclear cell infiltration around the necroses or in the periportal areas were noted. Post-necrotic fibrosis was present in the necrotic regions, especially in the subcapsular locations (figure 4). Capsular thickening of the spleen and, in the lungs, some alveolar oedema and cellular infiltration in the alveolar septa (figure 5) were the other principal observations.

Discussion

Infectious abortions of ewes and goats cause considerable economic loss. In addition to *Brucella* species, *Campylobacter fetus subsp. fetus*, *C. psittaci* (named more recently *C. abortus*), *Salmonella* and *Listeria monocytogenes* have all been isolated from abortion cases in Turkey [3, 11, 25, 33, 34, 36]. Aborted ewes were also found to be seropositive for *Coxiella burnetii* [17, 28]. Seropositivity rates for chlamydiosis among sheep and goats in different regions in Turkey were reported as varying from 1.6 to 29.3% [9, 13, 18, 19, 24, 27]. TÜRÜTOĞLU and ERGANIŞ [33] isolated *C. psittaci* in 13 of 98 (13.3%) aborted sheep foetuses, while TÜRÜTOĞLU et al. [34] reported having isolated *C. psittaci* from the vaginal swab samples, and from foetal viscera in an ewe belonging to a sheep breeding facility with cases of abortion. GÜLER et al. [15] found chlamydial DNA in 7 of 94 vaginal swabs (7.45%) and in the stomach contents of 6 out of 172 (3.49%) foetuses. YILMAZ et al. [37] isolated *C. psittaci* in Angora goats, in whom the pathogen was grown from one foetus. The pathogen isolation rates in our study are similar to those indicated by TÜRÜTOĞLU and ERGANIŞ [33], and the chlamydial DNA positivity in 6% was close to the proportion reported by GÜLER et al. [15].

The clinical findings and macroscopic lesions of *C. abortus* infections are not pathognomonic. Similar lesions can be seen with other diseases like brucellosis, campylobacteriosis, coxiellosis, toxoplasmosis and salmonellosis. The specific diagnosis of the disease is possible, however, by laboratory
techniques [22, 31]. The possibility of cross-reactions in CFT (complement fixation test), which is widely used in serological diagnosis and the difficulties in distinguishing antibodies from vaccination and infection are a disadvantage. Also, the increase in serum antibody titers of animals following abortion must be considered in evaluating serological results [2].

Pathogen isolation in embryonated yolk sacs is easier than isolation in tissue cultures, which necessitates a laboratory infrastructure. Elementary particles are scanned for in MZN (modified Ziehl-Neelsen)-stained preparations from embryonated yolk sac membranes. Blind passages are performed in the absence of elementary particles [33]. As a result, a long time is needed to declare a sample negative. Also, false positive results caused by the presence of Coxiella and Brucella are possible [30]. All the positive results with MZN staining in the present study were confirmed by PCR, leading to a complete agreement between the 2 methods.

Molecular methods like PCR are used to identify C. abortus in clinical samples, allowing a rapid diagnosis without the need of a culture. It has been published that C. abortus and C. psittaci can be differentiated by RFLP analysis of PCR products obtained with primers specific to clone 8 of the C. abortus helicase gene, and that these primers allow species-specific identification [10, 26]. The amplified products of other genes have also been reported for species identification [6, 16, 32]. In the present study primers of the C. abortus helicase gene clone 8 were used but a standard C. psittaci 6BC strain also yielded positive bands. Analysis of PCR products by RFLP identified the isolates as C. abortus. There are reports on C. pecorum also being a possible cause of abortion in ewes and goats [5]. As a result, discriminating the two species in abortion material is necessary. The specificity of clone 8 primers allows discrimination between C. abortus and C. pecorum [26]. The present study also showed that PCR-RFLP with clone 8 primers is adequate for the species identification of C. abortus. WANG et al. [35] who used CfoI, HaeIII, NdeI, Svu and Alu1 as enzymes for RFLP published that CfoI and HaeIII identify C. abortus isolates, while the large number of fragments obtained with Alu1 had been an obstacle to discriminating among different strains. The enzymes used by SIARKOU et al. [32] in RFLP were BfaI, SfcI, HpaI, BclI, DdeI and Accl; the authors report that BclI, SfcI and DdeI are the most reliable of these. BERRI et al. [6] identified C. abortus by breaking the amplified DNA fragments of the ompI gene with the AluI enzyme and the DNA products of the 16S-23S rRNA sequence using PsI and BglII. On the other hand, several investigators state that both the sensitivity and the specificity of RFLP using the AluI enzyme are high [5, 14, 16, 22, 26]. In the present study, the pathogen species may also be identified using HaeIII and AluI for RFLP, leading to the conclusion that both enzymes are specific enough in differentiating C. abortus from the other species.

C. abortus DNA can be identified by PCR in vaginal swab, aborted foetus, placenta and similar samples. Even though PCR is faster than culture, false negative results are possible because of the presence of cross-contamination and of factors inhibiting PCR [2]. In the present study, C. abortus DNA was identified in seven samples from yolk sac following inoculation to chicken eggs, while only 5 of the organ samples were positive, leading to speculation about factors that inhibit PCR in foetal samples. There are certain advantages to use vaginal swab samples for diagnosis. The risk of human contagion is significantly reduced thanks to the lack of direct contact with the abortion material and a very small amount of sample is sufficient. There are reports of large amounts of pathogen in the vaginal exudate from a few days before the abortion until a short time thereafter [22]. C. abortus DNA was found in six vaginal swab samples from two herds in which abortions had been observed, while the other swab samples from these same herds were negative, and the foetuses samples of the 2 positive herds were also positive. In the present study, the swab samples were collected a few days, sometimes 1-2 weeks following the abortion. The fact that the foetuses were positive while some vaginal swabs were not could have been due to a low number of pathogens, insufficient for PCR determination. The typical pathological lesions in a C. abortus infection are observed in the cotyledons and the placental membranes [8]. Due, however, to the fact that only the aborted foetus is sent to the laboratory for diagnosis, the liver, spleen and lungs of the foetuses were rarely examined. Even though the lesions were not specific, the presence of focal necrosis and periportal cellular infiltrate in the liver and of alveolar septal thickening in the lung is similar to the findings of other authors [20, 21].

As a conclusion, the importance of the role played by the C. abortus infection in the abortions seen in small ruminants in Turkey has been underlined. Vaccination in the areas where the disease is observed could potentially be beneficial. Infection by C. abortus is generally not immediately considered as a cause when abortion is seen in animals. Abortion products must be studied also for the presence of C. abortus in routine diagnosis laboratories. Due to the difficulties in isolating the pathogen, molecular methods should be used for both diagnosis and epidemiological investigation. In our opinion, large-scale epidemiological studies are needed to establish the extent of animal chlamydiosis in Turkey.

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