Identification and genotyping of *Chlamydia abortus* with MLVA from ruminant abortions in the Marmara region of Turkey

Mehmet Engin MALAL¹* Suheyla TURKYILMAZ²

Abstract

Abortion in ruminants is an important problem in Turkey and it leads to serious economic losses for farmers. The aim of this study is to diagnose *Chlamydia abortus* in ruminant abortions in the Marmara Region of Turkey and to determine the genotypes of the agent and obtain the first national epidemiological data in this sense. For this purpose, a total of 730 abortion materials (fetal tissue, fetal stomach contents, placentas, cotyledons, vaginal swabs) belonging to 267 cattle, 380 sheep, 70 goats and 13 water buffaloes were examined for *C. abortus* with species specific real-time PCR. DNAs of positive samples were genotyped by the Multilocus Variable Number Tandem Repeat Analysis (MLVA) method. From 730 materials, 87 (11.9%) were found positive for *C. abortus*. Positivity rates were 21.4% in goats, 16.6% in sheep, 7.7% in water buffaloes and 3% in cattle. The dominant genotype was found to be MLVA genotype 2 (93.1%), and 4 different genotypes including genotypes 3, 4, and 5 were involved in infections. So it is concluded that genotypic diversity of *C. abortus* is high in the Marmara region. It was also revealed that *C. abortus* was responsible for a significant proportion of small ruminant abortions in this region.

Keywords: *Chlamydia abortus*, Genotyping, MLVA, Real Time PCR, VNTR, Turkey

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Introduction

Chlamydia abortus causes an abortigenic disease called “chlamydiosis” or “enzootic abortion” in ruminants, especially in sheep and goats (Szeredé and Bacsadi 2002; Givens and Morally 2008). The disease is characterized by abortion in the last 2-3 weeks of pregnancy, premature birth, low birth weight and neonatal death within 48 hours after birth (Givens and Morally 2008).

The disease repeats periodically in the herd and is very difficult to control. When factors such as loss of offspring due to the disease, treatment expenses and decrease in milk production are taken into account, the disease causes significant economic loss (Longbottom et al., 2002, Carter and Wise 2004). The disease also poses a risk to public health (Ward 2006). The causative agent is the most important cause of sheep and goat abortions in many regions of the world and shows an endemic course (Essig and Longbottom 2015).

The prevalence of the disease is similar in Turkey’s neighbors as well. In molecular studies, it has been revealed that very high proportions (35.8%) of small ruminant abortions in Bulgaria are caused by C. abortus (Simeonov and Chilingirova 2018). In Iran, C. abortus has been detected in 11.0% to 38.0% of the small ruminant abortions and 7% of the disease causes significant economic loss (Longbottom et al., 2002, Carter and Wise 2004). The disease also poses a risk to public health (Ward 2006). The causative agent is the most important cause of sheep and goat abortions in many regions of the world and shows an endemic course (Essig and Longbottom 2015).

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C. abortus is genetically very homogeneous. The most preferred methods for genotyping C. abortus are Multilocus Sequence Typing (MLST) and Multilocus Variable Number Tandem Repeat Analysis (MLVA). When these methods are compared and contrasted, while MLST method can classify C. abortus into 5 different genotypes, 7 different genotypes can be obtained with MLVA (Siarkou et al., 2015). In addition, the MLVA method is suitable for direct typing from abortion material (Laroucau et al., 2009).

In Turkey, general serological studies have been carried out for this disease. Relatively high seropositivity rates have been seen in sheep such as 20.8% in Duzce (Karagül et al., 2019) and 32.0% (Öztürk et al., 2016) in Burdur. These results can be attributed to the agent as there are no vaccinations carried out in Turkey. Although the existence of the agent has been revealed by molecular studies and isolation studies, these studies far from present sufficient data on the prevalence of the disease in the national sense. The first study on the isolation of C. abortus in Turkey was carried out by Turutoglu and Erganis (1996) and it was reported that 16.3% isolation was obtained from sheep abortion materials. Güler et al., (2006) and Kalender et al., (2013) found 7.4% and 9.8% positivity from sheep abortions with the help of conventional PCR. There is no data regarding C. abortus genotypes in Turkey. However, it may be thought that the genotypic diversity was high due to its geographical location. The aim of this study is to diagnose C. abortus from ruminant abortions in the Marmara region of Turkey by real-time PCR and genotype with MLVA.

Materials and Methods

Ethical considerations: Ethical approval for the study was obtained from the Local Ethics Committee for Animal Experiments, Pendik Veterinary Control Institute (RPN 07/2018), Istanbul, Turkey.

Samples: In the study, samples from ruminant abortion cases obtained between 2017-2019 in the provinces of the working area of Istanbul Pendik Veterinary Control Institute (Bilecik, Istanbul, Edirne, Canakkale, Tekirdag, Kirkkareli, Bursa, Balikesir, Duzce, Yalova, Kocaeli, Sakarya) (Figure 1) were used.

The material of the study consisted of fetal stomach contents and internal organs of the fetus (lungs, liver, kidneys and spleen), placental tissue and vaginal swabs. For this purpose, 267 cattle, 380 sheep, 70 goats and 13 water buffalo samples belonging to 730 abortion cases were analyzed.

Figure 1  Provinces where samples were taken.
DNA extraction and real-time PCR: DNA extraction was performed with DNeasy® Blood & Tissue Kit (Qagen), according to the manufacturer’s directions. Fetal tissues and stomach contents, placental tissue and vaginal swabs were used for DNA extraction. Real-time PCR analysis was performed as previously described (Pantchev et al., 2009). Selected primers which are specific to C. abortus DNA were utilized for the amplification of the ompA gene region. Each reaction mix contained 0.9 μM of each primer, 0.2 μM of probe and 2 μl of template DNA. The amplification cycle included 15-minute denaturation at 95°C followed by 45 cycles at 95°C for 15s and 60°C for 60s. DNA of C. abortus S26/3 reference strain was used as the positive control and nuclease-free water as the negative control.

MLVA: MLVA was performed with template DNAs of the samples found positive with real time PCR. For MLVA, Laroucau et al., (2009)’s method had been modified. The reaction was carried out with 10 μl 2x mastermix (Norgen, Canada), 1 μl 10 qmol primers, 5 μl nuclease-free water and 3 μl template DNA in a total of 20 μl mix. The temperature time profile was 95°C 15 m of denaturation followed by 40 cycles at 95°C 30s, at 58°C 30s, at 72°C 45s, and final extention at 72°C 10 m. Five VNTR loci, (ChlAb 300, ChlAb 457, ChlAb 581, ChlAb 620, and ChlAb 914) were used as the genotyping markers. Primers described previously for MLVA were used. However, the 5’ ends of the forward primers were marked with Fam-6. After PCR, products were stored at -20°C until capillary gel electrophoresis. Capillary gel electrophoresis was performed to obtain amplicon sizes at Pendik Veterinary Control Institute using ABI 3130 XL (Applied Biosystems). The number of tandem repeats of 5 gene regions was found and MLVA genotypes were identified (Laroucau et al., 2009).

Statistical Analysis: The Pearson Chi-square test was run for both small ruminants and all the samples to identify whether there was a significant difference between provinces of the Marmara Region, Turkey. For the analysis, SPSS 18 (Statistical Package for Social Sciences) was utilized.

**Results**

Real Time PCR: C. abortus DNA was detected by real-time PCR from 87 (11.9%) of the 730 materials. 15 of 70 (21.4%) goat samples, 63 of 380 (16.6%) sheep samples, 1 of 13 (7.7%) water buffalo samples, 8 of 267 (3.0%) cattle samples were found to be positive. Real Time PCR results according to province and animal species are given in Table 1.

<table>
<thead>
<tr>
<th>Province</th>
<th>Sheep Positive</th>
<th>Goats Positive</th>
<th>Cattle Positive</th>
<th>Water Buffalo Positive</th>
<th>Total Sample</th>
<th>Total Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balikesir</td>
<td>37</td>
<td>6</td>
<td>2</td>
<td>-</td>
<td>71</td>
<td>7(9.8)</td>
</tr>
<tr>
<td>Bilecik</td>
<td>9</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>17</td>
<td>1(5.9)</td>
</tr>
<tr>
<td>Bursa</td>
<td>58</td>
<td>7</td>
<td>11</td>
<td>1</td>
<td>97</td>
<td>9(9.3)</td>
</tr>
<tr>
<td>Canakkale</td>
<td>81</td>
<td>16</td>
<td>21</td>
<td>2</td>
<td>143</td>
<td>19(13.3)</td>
</tr>
<tr>
<td>Duzce</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>32</td>
<td>41</td>
<td>4(7.7)</td>
</tr>
<tr>
<td>Edirne</td>
<td>85</td>
<td>16</td>
<td>6</td>
<td>3</td>
<td>157</td>
<td>21(13.4)</td>
</tr>
<tr>
<td>Istanbul</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>15</td>
<td>4(26.7)</td>
</tr>
<tr>
<td>Kırklareli</td>
<td>49</td>
<td>11</td>
<td>14</td>
<td>5</td>
<td>80</td>
<td>16(20)</td>
</tr>
<tr>
<td>Kocaeli</td>
<td>14</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>37</td>
<td>3(8.1)</td>
</tr>
<tr>
<td>Sakarya</td>
<td>7</td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>18</td>
<td>0(0)</td>
</tr>
<tr>
<td>Tekirdag</td>
<td>25</td>
<td>2</td>
<td>2</td>
<td>19</td>
<td>46</td>
<td>3(6.5)</td>
</tr>
<tr>
<td>Yalova</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>1(10)</td>
</tr>
<tr>
<td>Total</td>
<td>380</td>
<td>63(16.6%)</td>
<td>70</td>
<td>15 (21.4%)</td>
<td>730</td>
<td>87(11.9)</td>
</tr>
</tbody>
</table>

The results of the Pearson Chi-square test indicated that there was no significant difference between cities and small ruminants and also between cities and all of the animals (Table 2).

Genotyping: Genotypes of all 87 C. abortus positive samples were determined by MLVA (Figure 2). 81 out of 87 (93.1%) samples were found to be genotype 2. Genotype 2 was followed by genotype 5 with 4 (4.6%) samples, genotype 4 with 1 (1.1%) sample and genotype 3 with 1 (1.1%) sample. Genotypic classification according to animal species is given in Table 3.

Table 2  Statistical evaluation of positive results.

<table>
<thead>
<tr>
<th></th>
<th>χ² value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provinces and Positivity (Small Ruminants)</td>
<td>13.326</td>
<td>0.273</td>
</tr>
<tr>
<td>Provinces and Total Positivity</td>
<td>15.540</td>
<td>0.159</td>
</tr>
</tbody>
</table>
Figure 2  MLVA genotyping results
Discussion

Abortions caused by infectious agents in ruminants cause significant economic loss. Although the leading abortigenic agent in Turkey is Brucella spp., serological studies have shown that C. abortus may also be of high importance (Ozturk et al., 2016; Karagül et al., 2019). Different methods are used in the diagnosis of the disease but detection of Chlamydiaceae can be carried out more specifically by DNA-based methods (Longbottom et al., 2001). One of the preferred methods to get the most reliable and rapid results is real-time PCR (Sachse et al., 2009). In this study, the real-time PCR method, which can identify C. abortus as species-specific and which has a low detection limit, was preferred (Pantchev et al., 2009). Eighty-seven (11.9%) positive results were obtained from a total of 730 samples. The highest positivity rate was found in goats with 21.4% and in sheep with 16.6%. When the previous studies, including small ruminants in Turkey are examined, Türütoolg and Erganis (1996) isolated C. abortus from 16.3% of the sheep abortion materials. This finding is in line with our study. There have been some studies which also used the conventional PCR method. Guler et al., (2006) detected Cabortus DNA in 7.5% of the vaginal swabs taken from 15 sheep flocks. Kalender et al., (2013) detected 9.8% chlamydial DNA in 64 sheep abortions in North Anatolian Turkey. The positivity rates in sheep in our study are higher than those of the two studies. This difference might stem from the sampling way, analysis method used and the regions.

When the studies carried out in contexts other than Turkey are taken into account, in a study conducted in Switzerland, 39% of sheep abortions and 23% of goat abortions were found to have originated from C. abortus (Chanton-Greutmann et al., 2002). While the percentage belonging to goats in our study, is very close to their result (21.4%). The percentage was lower in sheep (16.3%). In the Iranian context, one of Turkey’s neighbours, different positivity rates (11-37%) were obtained from sheep and goats by PCR (Ebadi et al., 2015; Barati et al., 2017; Heidari et al., 2017). It can be said that in these contexts positivity rates are generally higher than the ones in our study. Abortion cases in sheep and goats between 2013 and 2018 in Bulgaria, another neighbour of Turkey, were investigated with PCR for C. abortus and 35.8% positivity was found. The positivity rates in sheep and goats were 41.9% and 25%, respectively (Simeonov and Chilingirova, 2018). In Bulgaria, the results belonging to goats are close to the ones in our study; however, the positivity rates of sheep are a lot higher.

In our study, cattle and water buffalo samples were also investigated. The positivity rate was 3% in cattle while C. abortus DNA was detected in a single water buffalo sample. In a study, Temur et al., (2007) determined the agent (5.5%) with the modified Ziehl-Neelsen staining method following egg inoculation from cattle abortions in Erzurum, Turkey. Kılıç et al., (2010) determined the DNA of the agent in 6.3% of the bovine abortions. Aras et al., (2017) determined 3% C. abortus positivity by the PCR method from bovine abortion cases in Konya and Aksaray, Turkey. Positivity rates in bovine abortions in our study showed similarity with these studies. Similar to the results of our study, in cattle abortion cases in Switzerland between 2003-2004, C. abortus was detected molecularly in 12 of 235 samples (5.1%) (Borel et al., 2006). Osman et al., (2012) investigated the presence of Chlamydiaceae in cattle in Egypt and identified C. abortus DNA in 15.1% of 73 cattle samples. This rate was higher than the cattle positivity in our study (3.0%).

Although Chlamydia species are known to infect water buffaloes (Burnard and Polkinghorne, 2016; Galiero, 2007), studies using molecular detection are limited. Greco et al., (2008) diagnosed Chlamydia spp. by nested PCR in a flock with an abortion rate of 36.8% and detected in 21.4% of 14 vaginal swab samples and 42.9% of 7 fetuses. It was revealed that two of the fetuses were simultaneously infected with C. abortus and Chlamydia pecorum and one was infected with C. abortus only. In Egypt, 9.8% of 102 water buffalo samples were found to be positive (Osman et al., 2012).

Although the number of water buffalo samples (13) in our study makes it impossible to compare the results with other studies. However, this study is important in terms of the fact that C. abortus was detected in a water buffalo fetus for the first time in Turkey.

When sheep and goats, which are the most affected species, were evaluated together, C. abortus DNA was detected in 78 of 450 materials (17.3%). This rate falls to 3.2% in cattle and water buffaloes together. These rates show that the disease is more important in small ruminants both in the Marmara Region and the world (Longbottom and Coulter, 2003). C. abortus was detected in Istanbul, Edirne, Kırklareli, Tekirdağ, Canakkale, Kocaeli, Yalova, Bilecik, Bursa and Balıkesir provinces. The only province in which there was no positive result was Sakarya. However, it was evaluated that the negative result could be misleading because of the number of materials provided (18). Additionally, the majority of them (10) were cattle samples with low positivity rates. The difference between positivity rates and provinces was not statistically significant, which shows that there is not a
single province which is affected by the disease more severely than the others; in fact, the whole region is affected by the disease.

It was observed that 81 of 87 (93.1%) C. abortus DNA genotyped in the Marmara Region was genotype 2. From 199 vaginal swabs belonging to sheep and goats in Algeria, 6.5% C. abortus positivity was found and all of them genotyped as genotype 2 with MLVA (Merdja et al., 2015). Yak abortion cases in China were investigated for C. abortus; 23.8% positivity was found in 9 abortion fetuses and 126 vaginal swabs and all of them were found to be MLVA genotype 2 (Li et al., 2015). In France, 88.9% of 9 C. abortus strains isolated from clinical samples were found to be genotype 2. In contrast, only genotype 4 and genotype 5 have been identified in the UK so far (Laroucau et al., 2009). These studies generally show that the dominant MLVA genotype in the world is genotype 2 and these results are also compatible with our study. However, in our study, it was observed that genotypes 3, 4 and 5 also play roles in infections in the Marmara Region, so it can be concluded that C. abortus has high genotypic diversity in Turkey.

It was observed that the dominant genotype is genotype 2 in cattle, sheep and goats. 95.2% of 63 sheep, all 16 goats and 75% of 8 cattle samples were classified as genotype 2. The only positive water buffalo sample was found to be genotype 3. Since there is only one positive sample, an assessment of the common genotype in water buffalo infections is not possible. However, it is remarkable that a single water buffalo isolate was found to be genotype 3, which is not a common genotype and no genotype 3 was detected in any other animal species. Due to the fact that we didn’t encounter any other studies of genotyping of water buffalo isolate with MLVA, we could not make a comparison. One cattle sample was found to be genotype 4 and one to be genotype 5. While genotype 4 was not detected from sheep and goat samples, 3 sheep samples were classified as genotype 5. From this aspect, strains found in the Marmara Region as genotypes 3 and 4 were detected only in large ruminants. In the previous studies, it was observed that the strains identified as genotype 4 and 5 were isolated in England, Germany and Greece (Laroucau et al., 2009). Genotype 6 to which Greek variant strains belong (Laroucau et al., 2009) has not been identified in the Marmara Region.

The regional prevalence of Chlamydia abortus in ruminants was shown for the first time in Turkey with Real Time PCR. The results show that C. abortus is commonly identified in small ruminants and it is responsible for a significant part of the abortions in the Marmara Region. Therefore, control and measuring strategies including vaccination should be established to decrease the abortion level. There is insufficient data on the status of C. abortus infections in humans in Turkey. Considering the prevalence of the disease and the zoonotic potential of the agent (Buxton et al., 2002; Pospischil at al., 2002; Walder et al., 2005; Pichon et al., 2020), some of the human abortion cases may be caused by C. abortus. For this reason, C. abortus research should be conducted in human abortions with ruminant contact in the Marmara Region.

This study is significant in many aspects. C. abortus strains were genotyped for the first time with this study in Turkey. Although the dominant genotype was found to be genotype 2, the existence of genotypes 3, 4 and 5 confirmed the high genotypic diversity of C. abortus in the Marmara Region, Turkey. More genotypes can be identified with studies that will be conducted in other regions of the country as well. Secondly, with this study, C. abortus was diagnosed for the first time from water buffalo in Turkey. Moreover, C. abortus which caused a water buffalo abortion was genotyped with MLVA for the first time in the world, and this work may guide future studies.

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