ISOLATION AND BIOTYPING OF **BRUCELLA MELITENSI S** FROM ABORTED SHEEP FOETUSES IN TURKEY

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Abstract

Aborted sheep foetuses were collected during the lambing seasons of 2004 to 2006. *B. melitensis* was isolated from 25 (29.76%) of 84 lungs and stomach contents. Based on the biochemical tests and agglutination test with monospecific A and M antiserum, the isolates were determined as *B. melitensis* biotype 3. The isolation of the *B. melitensis* from aborted sheep foetuses may show the importance of this agent in the aetiology of ovine brucellosis in Turkey. The determination of dominant biotype of *B. melitensis* responsible for ovine brucellosis is also expected to help to understand the epidemiology of ovine brucellosis in detail in this region and neighbouring countries.

**Key words:** sheep, *Brucella melitensis*, biotype, Turkey.

Ovine and caprine brucellosis is a zoonotic infection, which has important effects for both human health and animal reproduction. The main aetiological agent of small ruminant brucellosis is *Brucella melitensis* (1, 4). The predominant symptoms of *B. melitensis* infection are reproductive disorders such as abortion, stillbirth, delivery of weak offspring, and placenta retention in females, and orchitis and epididymitis in males (2, 3, 19). Abortion occurs during the last two months of gestation. While the disease has been eradicated in most industrialised regions, its occurrence is increasing in developing countries (11, 17), particularly in some Mediterranean and Middle Eastern countries (5, 15, 19). Available information indicates that *B. melitensis* infection is mostly widespread in Egypt, Sudan, Syria, Morocco, Turkey, Greece, Spain, and Italy, and in some Latin American countries. It is also common infection of animals and humans in countries neighbouring Turkey (5, 15, 19). *B. melitensis* has been reported to be the primary agent responsible for sheep abortions in Turkey (9, 10, 12, 16).

Based on the climate and geography, Turkey consists of seven different regions. These regions are very different from each other causing a variation in the epidemiology of infectious diseases. Ovine brucellosis due to *B. melitensis* is a significant problem for both public health and animal production in Turkey (22). Serological surveys for brucella antibodies in sheep indicate that 0.06% to 15.8% of the animals are infected (8, 16, 18). However, little efforts have been made to isolate *Brucella* sp. from cases of abortion in sheep (9, 12). This agent was isolated from human brucellosis cases in this region (22). The transmission of *B. melitensis* to cattle, buffaloes, and camels is now the predominant cause of brucellosis in animals and humans in most Middle Eastern countries. This situation increases the importance of *B. melitensis* as the causative agent of brucellosis (2, 3, 17, 19).

The diagnosis of brucellosis in sheep and goats is based on serological, bacteriological, allergic, and molecular methods (22). The most powerful mean to confirm the infection is bacteriological diagnosis since its specificity is much higher than that of other diagnostic methods. The gold standard that confirms the disease is the isolation of the bacterial agent. The existence of different *Brucella* biotypes among the *Brucella* species facilitated the identification of the source of the infection (12). Because of the complications involved in the diagnosis of the disease, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests, bacteriological isolation and identification of biotypes of the aetiological agent are necessary steps in design of epidemiological and eradication programmes (19, 23). Molecular diagnostic methods are also currently being used for the detection of *Brucella* sp. in various materials.

Bacteriological diagnosis, based on the isolation and identification of *Brucella* sp., is far from being routinely performed in many countries. However, the most powerful means to confirm the infection is still the isolation of the agent. Moreover, the identification of a biovar of *Brucella* strains is an epidemiological indication that can help to investigate the origin of a given brucellosis outbreak. Standard cultural methods for the diagnosis of brucellosis have also disadvantages, such as difficulties and time consumption in the isolation of this fastidious agents and biohazardous
potential of these species in laboratory environment (2, 7, 15, 17). There are limited studies on the biotyping of \textit{B. melitensis} isolated in Turkey (9, 12, 10, 21).

The present study aimed to isolate \textit{Brucella} sp. from aborted sheep foetuses by using standard cultural methods, and to biotype these isolates in order to establish a epidemiological base for studies on the control and prevention of brucellosis in this region.

**Material and Methods**

**Foetus samples.** Eighty-four aborted sheep foetuses were collected during the lambing seasons from 2004 to 2006. Each of the samples was submitted for diagnosis of abortion reason from different flocks in distinct locations in the Kars province and East Anatolia. The aborted foetuses were collected during the visits to the farms after the report of ovine abortion cases or the samples were directly submitted to the laboratory.

**Bacteriological examinations.** Small pieces of the lungs of the aborted foetuses were flame sterilised to sterilise the surface. Approximately 1 ml of foetal stomach contents was collected by aspiration using a sterile syringe. The samples from stomach content and a cut surface of the lung specimens were rubbed onto the 7% defibrinated sheep blood agar (Oxoid, CM 271) and onto Brucella agar without selective supplement (Oxoid, CM169). The inoculated plates were incubated at 37ºC in the presence 5%-10% CO₂ (candle jar) for up to 7 d. After the incubation, the suspected colonies were examined for \textit{Brucella} sp. \textit{Brucella}-suspected colonies were characterised by the morphology, positive Gram stain, oxidase, catalase, and urease production, nitrate reduction, growth on Mac Conkey agar, and indol test. In addition, apart from a rapid slide agglutination test, Tbilisi phage typing was performed using routine test dilution (RTD) (9).

**Biotyping.** The CO₂ requirement was determined immediately after the primary isolation. Then, \(H_2S\) production and growth in the presence of thionin, and basic fuchsin (20 µg/mL) dye incorporated into tryptic soy agar were tested. The agglutination with mono-specific A and M antisera was additionally performed. In order to discriminate isolates from \textit{B. abortus} biotype 2 and \textit{B. suis}, the growth on safranin O (100 µg/mL) was determined and the growth test on the media containing streptomycin (2.5 µg/mL) was performed to discriminate the isolates from vaccine strain Rev1 (9).

\textit{Brucella melitensis} vaccine strain Rev1 and \textit{B. melitensis} biotype 3 Ether (NCTC 10316), and \textit{B. abortus} biotype 2 86/8/59 (NCTC 10501) were used as standard strains.

**Results**

**Brucella isolation.** \textit{Brucella} sp. was isolated from the lungs and stomach contents of 25 (29.76%) out of 84 aborted sheep foetuses. Isolation rates for each year were shown in Table 1.

The colonies grown after incubation for 4-5 d were smooth, transparent, and dewdrop-like when inspected with a light source. Gram-negative coccobacilli were observed after Gram staining of these colonies. The isolates that in biochemical tests gave the results identical to those characteristic of \textit{Brucella} sp. were further examined with agglutination test using \textit{Brucella} A+M anti-serum and the agglutination was observed for all these isolates. Based on these results, 25 isolates were identified as \textit{Brucella} sp.

**Species identification and biotyping.** Not all the isolates required CO₂ after passages following the first isolation and they did not produce \(H_2S\) when analysed within 4-d incubation with filter papers containing lead acetate. After 5-d incubation, the isolates were able to grow on media plates containing thionin and basic fuchsin. All the isolates were determined as resistant to lysis by Tbilisi phage. Based on these properties, all these isolates were identified as \textit{B. melitensis}. The pure cultures of all the isolates showed agglutination with monospecific A and M antisera, therefore, these strains were determined as \textit{B. melitensis} biotype 3.

**Discussion**

Brucellosis is a worldwide zoonotic disease that is recognised as a major cause of heavy economic losses to the livestock industry and poses serious human health hazard (17). \textit{B. melitensis} is the main aetiologic agent of brucellosis in small ruminants. Ewes' and nanny-goats' aborted foetuses and products derived from sheep and goats remain the main source of infections. Ovine and caprine brucellosis were reported as a most common epidemic infection in Mediterranean and Middle Eastern countries, Asia, Latin America, and South Europe (15, 19).

**Table 1**

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of aborted foetuses analysed</th>
<th>Number of \textit{B. melitensis} isolations</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>14</td>
<td>4</td>
<td>28.57</td>
</tr>
<tr>
<td>2005</td>
<td>40</td>
<td>13</td>
<td>32.50</td>
</tr>
<tr>
<td>2006</td>
<td>30</td>
<td>8</td>
<td>26.66</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>25</td>
<td>29.76</td>
</tr>
</tbody>
</table>
The studies in various parts of Turkey indicate that the disease is widespread among sheep populations. Most surveys of brucellosis in Turkey rely on serological test only, without isolation of Brucella sp. and this can be misleading (6, 8, 13, 14). Confirmatory diagnosis must be provided by the isolation of aetiological agents. Therefore, the isolation of B. melitensis is important to study the epidemiology of brucellosis. The isolation of 25 B. melitensis strains from 84 aborted sheep foetuses may indicate very high prevalence of B. melitensis infection among sheep in this region and due to that, the disease may threat human and animal health.

The present study revealed that all of the 25 B. melitensis isolates were identified as biotype 3. Limited reports are available on the identification and biotyping of B. melitensis in Turkey. Sarısayın et al. (21) identified seven cases of biotype 2 and 2 of biotype 1 among 9 B. melitensis isolates. In a study performed in the Thrace area Erdoğan et al. (10) reported 25 cases of biotype 1 and 3 of biotype 2 among 29 B. melitensis isolates. Erdenliğ et al. (9) collected 78 B. melitensis isolates from various parts of Turkey, and 69 and 9 isolates were identified as biotype 3 and biotype 1, respectively. In a 3-year study performed in Central Anatolia, Guler et al. (12) found 37 cases of biotype 3 and 2 of biotype 1 among 39 B. melitensis strains isolated from aborted foetuses. The study from the same region reported 65 cases of biotype 3 and 5 of biotype 2 among 70 B. melitensis strains isolated from humans (22). Although various biotypes were reported from Turkey, the presented studies demonstrated that only biotype 3 was isolated. The determined biotype (biotype 3) in the present study is different from biotypes reported by Sarısayın et al. (21) and Erdoğan et al. (10). This may be explained by the differences in geography and time of those studies, and by eliminating some of the biotypes over years. Overall, the isolates from sheep of Eastern part of Turkey and Middle Eastern countries were dominantly determined as biotype 3, which is the result parallel to those obtained in the present study (19).

The resistance of Brucella sp. to Tbilisi phage and the application of biochemical tests on these agents are routinely used methods for the identification of Brucella sp. The isolates in the present study were not lysed by this phage. This phage resistance result and biochemical test results are in agreement with those reported by Erdenliğ et al. (9).

Brucellosis may be acquired directly through contact with contaminated material or aerosol infection or indirectly by grazing on contaminated pastures or through other materials. The traditional grazing management system of sheep and goats is still in use in this district. Moreover, several flocks belonging to different owners may graze the same pasture on the same day or the following day(s), which may spread the infection directly among herds. B. melitensis vaccine strain Rev 1 was isolated from some sheep abortions. The isolates in the present study were found to be different from B. melitensis Rev 1 and this finding may suggest that this vaccine strain is not the causative agent of ovine brucellosis in this region.

The isolation, identification, and biotyping of Brucella sp from aborted sheep foetuses by conventional cultural methods is difficult, time consuming, and potentially hazardous to laboratory workers. In spite of the availability of newly developed techniques, the isolation and identification of the agents by cultural techniques are still accepted as the gold standard for diagnosis of brucellosis. The isolation of the B. melitensis from aborted sheep foetuses in the present study may show the importance of this agent in aetiology of ovine brucellosis and abortions with a higher prevalence in this region comparing with other localities of Turkey. The determination of the dominant biotype of B. melitensis as biotype 3 responsible for ovine brucellosis in this region is expected to help to understand a detailed epidemiology of ovine brucellosis in the Kars district and neighbouring countries and to establish a base for the studies on the control and prevention of this important zoonosis.

References


