

## Isolation and biotyping of *Brucella melitensis* from aborted sheep and goat fetuses

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**Abstract:** The possible role of *Brucella* spp. in 65 abortion cases, 55 from sheep and 10 from goats, occurring in the birth seasons of 2004 and 2005 in northwestern Turkey was investigated. Colony morphology, agglutination by acriflavin, H<sub>2</sub>S production, CO<sub>2</sub> requirement, dye sensitivity in thionin, basic fuchsin, growth characteristics in streptomycin, lysis with *Tbilisi* phage, and agglutination with monospecific A- and M antisera were examined for identification and biotyping. The isolates from 21 of 55 sheep and 1 of 10 goat abortion cases were identified as *Brucella melitensis*. Biotyping revealed that 14, 6, and 1 of 22 *Brucella* spp. were *B. melitensis* biotype 3, *B. melitensis* biotype 1, and *B. melitensis* biotype 2, respectively. One strain isolated from a sheep abortion case was H<sub>2</sub>S- producing and was identified as atypical *B. melitensis*.

**Key words:** *Brucella melitensis*, sheep, goat, abortus, biotyping

### Atık koyun ve keçi fetuslarından *Brucella melitensis*'in izolasyonu ve biyotiplendirilmesi

**Özet:** Kuzeybatı Türkiye'de 2004-2005 yılları arasındaki iki kuzulama döneminde 55 adet koyun ve 10 adet keçi olmak üzere toplam 65 atık vakası *Brucella* spp. yönünden incelendi. İzolatların tür ve biyotip identifikasyonları için koloni morfolojisi, akriflavin ile aglütinasyon, H<sub>2</sub>S üretimi, CO<sub>2</sub> gereksinimi, tiyonin ve basik fuksin varlığında üreme, *Tbilisi* fajı ile lizis, streptomisinli besi yerinde üreme, monospesifik A ve M antiserumlarla aglütinasyon özellikleri incelendi. Çalışmada kullanılan 55 koyun atığının 21 adedi, 10 keçi atığının 1 adedi *Brucella melitensis* olarak tanımlandı. Biyotiplendirme sonucunda, 22 izolatın 14, 6 ve 1 adedinin sırasıyla *Brucella melitensis* biyotip 3, *B. melitensis* biyotip 1 ve *B. melitensis* biyotip 2 olduğu ortaya kondu. Koyun orijinli bir adet izolatın ise H<sub>2</sub>S üretimi yönünden pozitif olduğu saptandı ve atipik *B. melitensis* olarak değerlendirildi.

**Anahtar sözcükler:** *Brucella melitensis*, koyun, keçi, atık, biyotiplendirme

### Introduction

Ovine and caprine brucellosis is widespread around the world and the disease has an endemic distribution, especially in developing countries (1-4).

The prevalence of the disease in humans is mainly dependent on the animal reservoir, especially high rates of brucellosis in sheep and goats, and socio-economic situation of the countries (5). *Brucella*

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*melitensis* is the most commonly isolated species from humans (6). In Turkey, brucellosis is often encountered in cattle, sheep, goats, and humans (7-10). Although serological tests are used for monitoring the herds, bacteriological isolation is still the gold standard for definitive diagnosis of the infection or preparing eradication programs (11).

*Brucella* isolation and biotyping are carried out by standard procedures such as determination of CO<sub>2</sub> requirement, H<sub>2</sub>S production tests, and agglutination with monospecific antibodies. Growth in the presence of thionin and basic fuchsin is monitored for typing of *Brucella* cultures (12). *B. melitensis* has 3 biovars, besides several atypical strains, that do not conform to the classical identification patterns. The close relationship between the origin of the disease and epidemiology with atypical strains has been emphasized (13-16).

The main purpose of this study was to determine the presence and biotypes of *Brucella* spp. in sheep and goat abortion cases in northwestern Turkey.

## Materials and methods

**Samples:** Fifty-five sheep and 10 goat abortion cases that occurred during 2 birth seasons in 2004 and 2005 in different flocks in the towns of Yenişehir, İnegöl, Gönen, Mustafakemalpaşa, Karacabey, İznik, Manyas, and Susurluk, and the province of Bursa were evaluated. Breeds and rearing systems of the animals were not considered in this study. Fetuses were necropsied and the internal organs (lung, spleen, heart and liver) and abomasum contents were removed to obtain specimens. Abomasum contents were removed by searing an area of the stomach wall with a heated spatula, plunging the tip of a sterile injector through the seared area and transferring some of the contents and inoculated to Blood Agar and *Brucella* Selective Medium. Pieces from the internal tissues of aborted fetuses were collected with a set of sterile forceps and scissors and flamed after being plunged into ethanol. Tissues were dissolved in the sterile saline water at the same concentration and homogenized with an Ultraturrax (Micra RT-D9<sup>®</sup>).

***Brucella* antisera and reference strains:** Anti-*Brucella* polyclonal serum, monospecific A and M antisera, and the reference *Brucella* strains, i.e. *B.*

*melitensis* biotype 1 (16 M-ATCC 23456), *B. melitensis* biotype 2 (63/9-ATCC 23457), *B. melitensis* biotype 3 (Ether-ATCC 23458), *B. abortus* biotype 1 (544-ATCC 23448), and *B. melitensis* Rev 1 (vaccine strain), were provided by the *Brucella* Laboratory of Pendik Veterinary Control and Research Institute, İstanbul, Turkey.

**Bacterial isolation and identification:** In smears of fetal membranes and abomasum content, the red stained organisms against a blue background and gram negative coco-bacilli were sought by Modified Ziehl-Neelsen method and Gram stain, respectively. Blood Agar Base No. 2 (Oxoid<sup>®</sup> CM 271) containing 7% defibrinated sheep blood and *Brucella* Medium Base (Oxoid<sup>®</sup>-CM0169) containing *Brucella* Selective Supplement (Oxoid<sup>®</sup> SR83) were used for isolation. Abomasum contents and homogenates removed for culture were inoculated on agar plates and incubated in 37 °C and 5% CO<sub>2</sub> conditions for 3-8 days for growth. Colony morphology and opacity were detected by stereomicroscope (Olympus SZ61<sup>®</sup>). For distinguishing smooth and rough colony formation, a solution of neutral Acriflavine (Sigma<sup>®</sup> A 8126) was prepared at 1:1000 dilution freshly on the day, and a small amount of culture inoculated onto the loop of solution and the slide was examined under a low power stereomicroscope. The agglutination features of colonies were examined using anti-*Brucella* polyclonal serum.

**Biotyping of *Brucella* cultures:** Lysis by *Tbilisi* phage (at Rutin Test Dilution-RTD), requirement for added CO<sub>2</sub>, growth in the presence of thionin (20 µg/mL) and basic fuchsin (20 µg/mL), production of hydrogen sulfide, oxidase and urease features, and agglutination with monospecific anti-A, anti-M sera were investigated for biotyping (12,17). Growth features in a 2.5 mg/mL concentration of streptomycin were examined for differentiation between the field strain and Rev 1. Serum Dextrose Agar (SDA) (12) was used as base medium for dye and antibiotic susceptibility tests. Suspensions were prepared of bacterial and reference cultures by suspending a loopful of fresh culture in 1 mL of sterile saline. A sterile cotton swab was immersed in the bacterial suspension and 4 suspensions inoculated parallel to each SDA plate. For determination of susceptibility of phage lysis, 20 µL of *Tbilisi* phage at

$10^4 \times$  RTD was dropped into the inoculation zone. The oxidase test was performed by Kovaks' method. Urease activity of isolates was determined in Christensen's medium. All plates were incubated at 37 °C and 5% CO<sub>2</sub> conditions for growth after 3-4 days.

## Results

*Brucella* spp. were isolated from 33.8% of 65 aborted fetuses, 21 of 55 sheep and 1 of 10 goat aborted fetuses. The agent was isolated in both the abomasum content and internal organ homogenates in all cases. The colony morphology of all 22 isolates was smooth and all were negative in the agglutination test with acriflavine and positive to oxidase and urease. The lysis with *Tbilisi* phage at RTD and growth features in streptomycin were negative, and growth features in basic fuchsin and thionin were positive of all isolates. After biotyping tests and agglutination by polyclonal antiserum, all of the 22 *Brucella* isolates were identified as *B. melitensis*. Out of 21 *Brucella melitensis* isolates, 14 were identified as *B. melitensis* biotype 3 (66.6%), 6 were *B. melitensis* biotype 1 (28.5%), and 1 was *B. melitensis* biotype 2 (4.7%). One ovine isolate (number 6), which was identified as *B. melitensis* biotype 3 after testing positive with monospecific anti A and anti M sera, was ascribed as an atypical or unusual variant as it was H<sub>2</sub>S producing. One isolate from an aborted goat fetus was identified as *B. melitensis* biotype 1. The biochemical identification characteristics and biotypes of 22 *B. melitensis* isolates are shown in the Table.

## Discussion

Sheep and goat brucellosis caused by *B. melitensis* has a major impact on human health, besides causing significant economic losses in animal husbandry. *B. melitensis* is the most virulent biotype for humans (18). The disease is largely controlled by EU-supported eradication programs in France, Greece, Italy, Portugal, and Spain (2,4). It is endemic and has high prevalence in West Asia and North African countries (1,3). In Turkey, sheep and goat brucellosis is investigated by bacteriological, serological, and molecular methods and the occurrence of the disease is monitored by epidemiological studies (8,9,19). In a 10-year retrospective study by Güler et al. (8),

brucellosis was found to be the primary cause among all infectious abortus agents in sheep as evidenced by bacteriological and serological methods. In the present study, *B. melitensis* was isolated in 33.8% of 65 sheep (55) and goat (10) fetuses. This finding suggests that *B. melitensis* plays an important role in sheep and goat abortion cases in northwestern Turkey.

There are 3 biotypes of *B. melitensis*, which are isolated in different rates in different countries. *B. melitensis* biotype 3 is the most prevalent biotype in the Mediterranean and Middle East countries. Biotypes 1 and 2 are found to a lower extent in these regions, but are more common in southeastern Europe (1). In a previous study, Erdenliğ and Şen (7) revealed that *B. melitensis* biotype 3 (88.5%) and 1 (11.5%) are prevailing in Turkey. In addition, Güler et al. (9) detected the same prepotent biovar in aborted sheep fetuses. Our study confirms that *B. melitensis* biotype 3 is predominant in northwestern Turkey.

There are atypical variants of *Brucella* showing little correlation with standard biotyping procedures (13,15,16,20). Corbel (14) biotyped 500 *B. melitensis* isolates between 1980 and 1986 and found 29 isolates growing in basic fuchsin but not in thionin, which is incompatible with the standard biotyping procedures. As a result, it was emphasized by the same authors that the criteria of biotyping must be studied carefully again. Banai et al. (13) revealed the existence of atypical variants that are susceptible to penicillin, basic fuchsin, and thionin. These researchers claimed that atypical strains did not form a new taxonomic group but was a result of structural changes in Group 2 proteins (porin proteins). In this study, one isolate taken from sheep fetus showed typical characteristics of *B. melitensis* genus, but the H<sub>2</sub>S production feature was positive. The isolate identified as *B. melitensis* biotype 3 was characterized as an atypical or unusual strain. The identification procedures were performed 3 times for confirmation. In the previous studies (13,14,16) while the presence of *B. melitensis* atypical variants was attributed to different characteristics of their sensitivity to dyes and antibiotics, it is interesting to note that atypical or unusual *B. melitensis* isolated in this study was differentiated from the others in terms of H<sub>2</sub>S production.

Table. Identification and biotyping of isolates.

Isolate Number	Animal Species	Colonial Morphology	Agglutination with acriflavine	Catalase	Oxidase	Urease	H <sub>2</sub> S	Basic	Thionin Fuchsin	Streptomycin	Lysis by Tbilisi phage (RTD)	A	M	A + M	Biotype
4, 10, 17, 18, 19, 20*	Sheep Goat	Smooth	-	+	+	+	-	+	+	-	-	-	+	+	<i>B. melitensis</i> biotype 1
16	Sheep	Smooth	-	+	+	+	-	+	+	-	-	+	-	+	<i>B. melitensis</i> biotype 2
1, 2, 3, 5, 7, 8, 9, 11, 12, 13, 14, 15, 21, 22	Sheep	Smooth	-	+	+	+	-	+	+	-	-	+	+	+	<i>B. melitensis</i> biotype 3
6	Sheep	Smooth	-	+	+	+	+	+	+	-	-	+	+	+	Atypical <i>B. melitensis</i>

\*Goat isolate

Regarding the epidemiological aspects, it is important to emphasize the role of *B. melitensis* Rev 1 in abortion cases in countries like Turkey where sheep and goats are vaccinated widely. Isolation of *B. melitensis* Rev 1 from aborted fetuses and milk secretions of sheep and goats was reported in various studies (21,22). In this study, none of the 22 *B. melitensis* isolates matched a vaccine strain. This result shows that *B. melitensis* Rev 1 was not responsible for sheep and goats' abortion cases in the study area.

In conclusion, *B. melitensis* biotype 3 is the dominant strain and it has a major role in sheep abortions in northwestern Turkey. Since one atypical strain was also isolated in this study, genetic

characterizations of *B. melitensis* isolates by molecular typing methods may be helpful in future studies.

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