Conventional and molecular biotyping of *Brucella* strains isolated from cattle, sheep and human

Tuba İÇA¹, Fuat AYDIN¹, K. Semih GÜMÜŞSOY¹, Duygu PERÇİN², Ahmet Bülent SÜMERKAN², Fulya OCAK³, Seçil ABAY¹, H. Okan DOĞAN³, Arzu FINDIK⁵, Alper ÇİFTCI⁵

¹ University of Erciyes, Faculty of Veterinary Medicine, Department of Microbiology, Kayseri, ² University of Erciyes, Faculty of Medicine, Department of Microbiology, Kayseri, ³ University of Celal Bayar, Faculty of Science and Arts, Department of Biology, Manisa, ⁴ Ankara Numune Education and Research Hospital, Emergency Biochemistry Laboratory, Ankara, ⁵ University of Ondokuz Mayıs, Faculty of Veterinary Medicine, Department of Microbiology, Samsun, Turkey.

**Summary:** In this study, the role of *Brucella* spp. in cattle and sheep abortions among Kayseri region was investigated and predominant subspecies and biovars in this region were determined by conventional and molecular biotyping methods. For this purpose, 61 cattle and 64 sheep abortion material and also 50 human blood isolates were examined. A total of 29 *Brucella* spp. strains were found to belong to *B. abortus* biovar 3 and biovar 3b using both conventional and molecular biotyping methods, respectively. All sheep originated *B. melitensis* biovars strains and human originated *Brucella* spp. strains were found to belong to *B. melitensis* biovar 3 using both conventional and molecular methods. As a result, predominant biovars causing brucellosis in human, cattle and sheep in Kayseri, Turkey were detected. These findings were considered to be useful in prevention and controlling activities for Brucellosis in Turkey.

Keywords: Biotyping, *Brucella*, enhanced AMOS-ERY PCR.

**Introduction**

Brucellosis caused by an intracellular pathogen which is belong to *Brucella* genus is one of the most important zoonotic infections worldwide as well as in Turkey. *Brucella* spp. cause infections mainly characterized by abortion, infertility, mastitis, arthritis and orchitis in cattle, sheep, goats and pigs. The disease is also associated with the production losses relating to decreases in milk production and breeding value and infertility. Brucellosis constitutes an important public health problem usually resulting from the transmission via direct contact with infected animals and animal products.

The diagnosis of brucellosis is usually based on serology and culture. The identification of *Brucella* isolates at the species and biovar levels by classical bacterial methods is time consuming because *Brucella* spp. requires long incubation period and several phenotypical tests are needed to determine biovars. Also, infection risk in laboratory personnel who work for

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diagnosis of brucellosis by laboratory tests should not be forgotten. Although serological tests are useful to diagnose of the disease, cross-reactions with some other bacteria may lead to false positive results. The requirement of more rapid and sensitive diagnostic tests for brucellosis has come into question because of the importance of disease in terms of both public and animal health and in terms of economic importance. Currently, the diagnostic methods based on the detection of nucleic acid such as Polymerase Chain Reaction (PCR) are often used because such methods meet these requirements. The range of the identification of Brucella species has been expanded using the different versions of PCR. B. abortus biovar 1, 2 and 4, three biovars of B. melitensis, B. ovis and B. suis biovar 1 can be identified and differentiated by Brucella AMOS (Abortus-Melitensis-Ovis-Suis)-PCR assay based on the existence of repetitive IS711 copies in the genome of different Brucella species (7). However this method is not useful for identification of all subspecies and further tests are needed. New oligonucleotide primers have been added to the multiplex Brucella AMOS PCR assay and the ability of AMOS assay to identify more number of Brucella biovars and also to discriminate between B. abortus vaccine strains and wild-type isolates of Brucella has been expanded. A new method, known as AMOS-ERY PCR, involves the use of ery locus-specific primers and permits all Brucella species to be identified (8, 20). Ocampo-Sosa et al (20) have described a 5.4 kb deletion next to one of the IS711 copies in B. abortus biovars 5, 6 and 9 and also in some biovar 3 strains. However this deletion has not been detected in Tulya strain of biovar 3 of Brucella biovars and 5.4 kb fragment deleted in some field strains and biovars of B. abortus. The oligonucleotides used in this study are listed in Table 1. For the 20 µl of reaction mixture, 2µl of 10xPCR buffer, 0.2 mM of each dNTP, 1.5 mM of MgCl₂, 0.28 µl of IS711 primer and 0.04 µl of the rest of the primers, 1 U Taq polymerase and 1 µl extracted DNA were mixed. The amplification was carried out as follows: initial denaturation at 95°C for 10 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min and a final extension at 72°C for 5 min (6). Amplicons were loaded onto 0.8% agarose gel and containing 1 µg/ml ethidium bromide and electrophoresed at 100 V for 40 min.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5'–3')</th>
<th>Reference</th>
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<tr>
<td>PBo</td>
<td>CGGGTTCTGGCACTACCATCTGCGTCA</td>
<td>10</td>
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<td>PBo</td>
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<td>ERY1</td>
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<td>25</td>
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<td>ERY2</td>
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<td>25</td>
</tr>
<tr>
<td>DEL569</td>
<td>GCGCAGCGGTGCGGCAATTTA</td>
<td>25</td>
</tr>
</tbody>
</table>

**Results**

Isolation of Brucella spp.: Seventeen strains (27.9%) were isolated from 61 aborted bovine fetuses and 12 strains (18.7%) were isolated from aborted sheep fetuses.
Conventional identification and biotyping of Brucella spp.: While all bovine isolates (17 strains) were identified as *B. abortus*, all sheep and human isolates were identified as *B. melitensis* by conventional tests. In conventional biotyping of these strains, all *B. melitensis* strains and bovine *B. abortus* strains were found to belong to biovar 3.

Molecular identification and biotyping by Enhanced AMOS-ERY PCR: Molecular identification of all Brucella isolates at species level and molecular biotyping of these strains were performed by Enhanced AMOS-ERY PCR. A 1270 bp band was observed in all *Brucella* strains with Ery1-Ery2 primers, except for *B. abortus S19 strain*. In addition to this band (1270 bp), 731 bp, 498 bp, 1700 bp and 976 bp bands were observed for *B. melitensis*, *B. abortus* (biovar 1, 2, 4), *B. abortus* (biovar 3b, 5, 6, 9) and *B. ovis*, respectively. Field isolates were differentiated from *B. abortus S19* vaccine strain by observing two close bands which were approximately 500 bp with Ery 1-2 in S19 strain (Figure 1 and 2).

Sheep and human isolates of *Brucella* were identified as *B. melitensis* by Enhanced AMOS-ERY PCR. Bovine *Brucella* isolates were identified as *B. abortus* and these strains were found as biovar 3b.

Discussion

Brucellosis still poses a threat both to human and animal health in many countries despite having an eradication program. The detection of predominant species and biovar/biovars among infected human and animals is the major step to develop control strategies for Brucellosis. The prevalence of brucellosis varies from region to region throughout the country however this infection is widespread particularly in developing Mediterranean and Middle Eastern countries (1, 18, 21). In this study, the prevalence of *brucellosis* in abortus cases of cattle and sheep in Kayseri Province was investigated. It was determined by cultural and molecular techniques and the prevalence of brucellosis in abortus cases of cattle and sheep were detected as 27.9% and 18.7%, respectively. In a study conducted in Kars Province of Turkey, *Brucella* spp. has been isolated and identified from 37 out of 62 (55.6%) aborted cattle fetuses (25). Also, Ünver et al. (25) have isolated 38% of
B. melitensis from aborted sheep fetuses. In another study in the same region, Brucella isolation rate from milk and vaginal samples from cattle has been detected as 4.4% and 6.4%, respectively (12). Gülhan et al. (16) have detected the percentage of Brucella spp. isolation from aborted cattle fetuses as 26.7%. The isolation rate of Brucella spp. shows great variety between regions of Turkey, even in the same region. This may related to different management and climatic conditions, different sources of samples from which the isolation is performed.

Human brucellosis has serious public health consequences in endemic areas. Worldwide, among all Brucella species, Brucella melitensis is the most prevalent species causing human brucellosis. Especially in countries where animal brucellosis has not been able to control yet, due to inadequate food-safety measures, absence of effective hygienic control and laboratory safety, millions of human beings are at risk. Also Turkey is an endemic country for brucellosis (3, 13). According to reports from the Turkish Ministry of Health, 37 cases were reported in 1970, with numbers rising to 18 408 cases in 2004 (incidence rate 25.67/100 000). This increase is considered to be a result of improvements in the Kars province (23), most isolates (93.75%) were found to belong to biovar 3. Also in different regions of Turkey, Brucella melitensis isolates from various sources have mostly biotyped as biovar 3 (14, 15). The biotyping results obtained in this study comply with the results of previous studies mentioned above and show that the predominant biovar of both B. abortus and B. melitensis is biovar 3.

Bolca et al (4) have reported that 75.86% of B. melitensis strains that they were isolated from various samples of humans were typed as biotype 3, the remains were found as biotype 1 (13.79%) and were found to have rough colony morphology (10.34%). In a recent study (24), it has been reported that all Brucella strains isolated from human diagnosed with brucellosis in Central Anatolia Region of Turkey were identified as B. melitensis and 92.8% of them were conventionally typed as biovar 3. Therefore, the most prevalent type of B. melitensis from human brucellosis seems to be biovar 3.

Due to the close genetic similarity among Brucella species and the strains in Brucella genus, the differentiation of species and biovars is difficult by conventional methods. Furthermore, the instability for some of the phenotypic characteristics of Brucella that have been reported by Meyer (19), makes it difficult to identify some particular strains. Furthermore because it is very risky to handle live Brucella in terms of possible laboratory infection, for handling samples and live bacteria for eventual identification and biotyping, the level 3 biocontainment facilities and highly skilled technical personnel are required. In order to avoid these disadvantages, methods based on PCR are becoming very useful and to date considerable progress has been made in the development of more sensitive, specific, easier and cheaper PCR techniques for Brucella detection (26). It has been reported that several researchers have described various PCR assays for both diagnosing and typing of Brucella species (20). Among them a genus specific PCR firstly developed for Brucella have been unable to differentiate Brucella species. Then AMOS (from the initial letters of abortus, melitensis, ovis and suis) PCR assay developed by Brick and Halling (7) have been reported to be able to identify and differentiate most Brucella species. Because of inadequacy in
differentiation of all biovar and species new oligonucleotides have been added to AMOS-PCR primer cocktail to identify more number of Brucella biovars and also to discriminate between B. abortus vaccine strains and wild-type isolates of Brucella (AMOS-ERY PCR). Then, another specific primer, DEL569 designed for a 5.4 kb deletion next to one of the IS711 copies in B. abortus biovars 5, 6, 9 and in some field strains of biovar 3 of B. abortus has been added to the AMOS-ERY-PCR primer cocktail. Thus, AMOS-ERY-PCR has become more distinctive to discriminate B. abortus biovars 3b, 5, 6 and 9 from the rest of Brucella species and biovars and its name has been called as Enhanced AMOS-ERY-PCR. While Tulya strain and the field strains isolated from Africa have been suggested to belong to biovar 3a, the European field strains have been suugested to belong to group 3b (20). Ica et al. (17), have subtyped all 75 Brucella abortus strains isolated from aborted bovine fetuses in several regions of Turkey as B. abortus biovar 3b by enhanced AMOS-ERY PCR. Similarly in this study, all B. abortus strains isolated from bovine aborted fetuses were typed as biovar 3b using AMOS-ERY PCR. This result shows that B. abortus biovar 3b is the predominant subtype in Kayseri provinces of Turkey. Although some reports concerning the conventional biotyping of B. melitensis isolates originated from sheep materials, it has not been found any report concerning molecular biotyping of B. melitensis in Turkey. In this study all B. melitensis isolates were biotyped by a conventional method and described as biovar 3. Furthermore, human B. melitensis isolates were also identified as biovar 3 using the same technique. This shows that same biovar (biovar3) of B. melitensis is predominant in Kayseri.

As well as in many countries of the world, in Turkey a vaccination program is implemented to eradicate Brucellosis. For this aim, B. abortus S19 vaccine is used in cattle. In some cases, there are some complaints about vaccine associated infections. The reliable differentiation of vaccine strains from field isolates is an important element in brucellosis control programs. The conventional methods can not meet this requirement however B. abortus S19 vaccine strain is readily differentiated from field strains by Enhanced AMOS-ERY PCR. In this study, none of the bovine isolates were B. abortus S19.

In Kayseri Province, although the role of Brucella spp. was found in bovine and ovine abortus cases examined in this study, the rates of Brucellosis were relatively low (27.9% and 18.7% in bovine and ovine abortus cases, respectively). Further studies should be performed to detect other agents causing abortion. This is important to maintain breeding activities economically.

In conclusion, Enhanced AMOS-ERY PCR was found to be useful as a rapid, easy and discriminative method in this study. Besides conventional methods, Enhanced AMOS-ERY-PCR as a molecular biotyping method provided the identification of predominant biotypes of Brucella strains in Kayseri province of Turkey. To control brucellosis, the results of this study and further more detailed studies concerning the identification and characterization of dominant strains throughout the country are considered to be helpful, especially for vaccine development studies.

Epidemiological data obtained from this study may be useful to evaluate local situation of brucellosis in Kayseri and they may allow us to detect predominant biovar/biovars in this area. Especially latter is significant in prevent and control activities for brucellosis.

References
10. Büyü, F, Şahin M (2011): Investigation of Brucella species from various samples of aborted cattle in Kars Province (Turkey) by cultural and molecular methods and epidemiological analysis of cases, The Journal of the Faculty of Veterinary Medicine, University of Kafkas, 17 (5), 809–816.
12. Çelebi Ö, Ölu S (2011): Bacteriological and Molecular Description of Brucella Species&Isoalted from Milk and Vaginal Swab Samples of Aborted Cattlein Kars Region.
The Journal of the Faculty of Veterinary Medicine, University of Kafkas, 17 (1), 53–58.


Address for correspondence:
Dr. Arzu Fındık
Ondokuz Mayıs Üniversitesi
Veteriner Fakültesi
Mikrobiyoloji Anabilim Dalı
Kurupelit Kampüsü. Atakum, Samsun, TÜRKİYE.
E-mail: afindik@omu.edu.tr