

Conventional and molecular identification of *Brucella* isolates from livestock in Turkey

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Abstract: The main object of the study is to demonstrate that Bruce-ladder multiplex PCR, which is capable of identify all *Brucella* species including vaccinal strains, is a safe and practical method that is alternative to bacteriological culture methods. Brucellosis is a zoonosis of great socio-economic importance which is endemic in Turkey. In three-year period from 2009 to 2011, a total of 281 *Brucella* spp. isolates from cattle, sheep and goats were identified and characterized by both conventional biotyping procedures and the PCR based method called Bruce-ladder. Species identification from both phenotypic testing and Bruce-ladder was concordant for all isolates tested. The results showed preferred host pattern in *B.abortus* isolates. In terms of species identity, it was found that 94% of the isolations derived from cattle were *B.abortus* and were exclusively biotype 3. *B.melitensis* biotype 3 and biotype 1 were the prevalent biotypes in sheep and goats. Two isolates from sheep were identified as *B.melitensis* Rev.1 vaccine strain. Surprisingly, one of the goat isolates was identified as *B.abortus* S19 vaccine strain. Other interesting observations were the isolation of penicillin-sensitive non vaccine strains of *B.abortus* and *B.melitensis* as well as an isolation of an atypical *B.abortus* biotype 3 strain on the basis of growth inhibition in the presence of basic fuchsin dye. At the end of this study, it was concluded that monitoring *Brucella* isolates and identify them are indispensable epidemiological tool for brucellosis control

Keywords: Atypical *Brucellae*, bruce-ladder PCR, *Brucella* typing, Rev.1.

Türkiyede çiftlik hayvanlarından izole edilen *Brusella* izolatlarının konvansiyonel ve moleküler identifikasyonu

Özet: Çalışmanın başlıca amacı, izole edilen *Brusella* suşlarının identifikasyonunda, aşı suşları dahil tüm türleri saptayan Bruce-ladder multipleks PCR'nin bakteriyolojik kültür yöntemlerine güvenli ve kolay uygulanabilir bir alternatif sunduğunu göstermektir. Bruselloz, Türkiye'de yaygın olarak görülen ve büyük sosyo-ekonomik etkiye sahip bir zoonozdur. 2009 yılından 2011'e, 3 yıllık bir dönemde, sığır, koyun ve keçilerden izole edilen toplam 281 *Brucella* spp. izolatı, konvansiyonel biyotiplendirme ve PCR temelli Bruce-Ladder metodları birlikte kullanılarak tanımlandı ve karakterize edildi. Fenotipik test ve Bruce-ladder metodlarıyla tür identifikasyonu sonuçları, tüm izolatlar için birbirleriyle uyumluydu. Sonuçlar *B.abortus* izolatları için, tercih edilen konakları belirledi. Tür düzeyinde identifikasyona göre sığırlardan elde edilen izolatların %94'ü *B.abortus* ve çoğunlukla biyotip 3'tü. *B.melitensis* biyotip 3 ve biyotip 1, koyun ve keçilerdeki yaygın biyotiplerdi. Koyunlardan iki izolat, *B.melitensis* Rev.1 aşı suşu olarak identifiye edildi. Keçi izolatlarından biri, beklenmedik şekilde *B.abortus* S19 aşı suşu olarak tanımlandı. Diğer ilginç gözlemler, penisiline duyarlı olan ancak aşı suşu olmayan *B.abortus* ve *B.melitensis* suşları ile 3 adet Bazik fuksin boyasına duyarlı *B.abortus* biyotip 3 suşunun izolasyonlarıydı. Çalışmanın sonunda, *Brucella* izolatlarını izlemek ve onları tanımlamanın brusellozun kontrolünde vazgeçilmez bir epidemiyolojik araç olduğu kanısına varıldı.

Anahtar sözcükler: Atipik *Brusellalar*, bruce-ladder PCR, *Brusella* tiplendirme, Rev.1.

Introduction

Brucellosis is a zoonosis of global importance that causes reproductive problems in ruminants and serious

economic losses in the livestock industry (10). The genus *Brucella* currently consists of 11 species based on mainly host specificity, of which six (*B.abortus* (cattle),

B.melitensis (sheep and goats), *B.suis* (Swine but also hares, reindeer, rodents), *B.ovis* (sheep), *B.canis* (dogs), *B.neotomae* (wood rats)) are classical species. Newly described species include *B.ceti* (dolphins, porpoises), *B.pinnipedialis* (seals), *B.microti* (voles), *B.inopinata* (human breast implants) and *B.papionis* (baboons). *B.abortus*, *B.melitensis* and *B.suis* can be further subdivided into biotypes based on cultural, biochemical, and serological differences (10, 14, 19, 23).

From the previous studies centered on *Brucella* spp. characterizations in Turkey, it was shown that whilst brucellosis in sheep and goats was attributable to infection by either *B.melitensis* biotype 3 or biotype 1 (6, 11, 12, 16), brucellosis in cattle could be attributable to exclusively *B.abortus* biotype 3 (12, 18, 26).

In the case of control measures for brucellosis in endemic regions, it is advisable to vaccinate livestock to control the disease. *B.abortus* S19 and *B.melitensis* Rev.1 are the vaccine strains used to vaccinate cattle and sheep, and goats, respectively, in Turkey. Although vaccination offers protection from brucellosis in animals, sometimes these vaccine strains can cause abortions if especially administered to pregnant livestock (19).

Accurate identification of the *Brucella* species and biotypes is critical for the success of the control and eradication programs since it clearly demonstrates the prevailing *Brucella* species and biotypes in any given region or country as well as monitors of vaccine strains where vaccination is undertaken. Such information can also be used to trace and control newly introduced strains and it is indispensable for obtaining an accurate evaluation of the epidemiological status of herds and countries (7, 10).

Conventional biotyping procedures rely upon phenotypic differences seen in the culture of isolated bacteria and they include CO₂ requirement, H₂S and urease production, degrees of growth inhibition in the presence of certain dyes, agglutination with mono-specific antisera specific for A and M *Brucella* surface antigens, susceptibility to antibiotics and lysis by *Brucella* phages (2, 8). More recently, with advances in molecular techniques and a better understanding of genomes from a variety of *Brucella* species, several PCR-based assays have been published that can identify *Brucella* isolations to strain level as well as differentiate field strains from vaccine strains (5, 15, 20). *B.abortus* RB51 can be distinguished from field strains by serological means through its rough phenotype, but the smooth strain vaccines, *B.abortus* S19 and *B.melitensis* Rev.1 cannot be distinguished by this methodology. However, phenotypic characteristics and, more recently, molecular tests like Bruce-ladder can provide the means to identify these smooth vaccines from field strains. Although the use of these tests has been limited to identifying bacterial isolations, there is a potential use of these techniques

directly on DNA extractions from clinical materials, thus circumventing the need for bacterial isolation for characterization of the causative agent (22). Further, as molecular assays do not require viable organisms and can work directly from heat-inactivated material, they are safer than conventional tests and do not require the expert who works at handling facilities required to work with live *Brucella* cultures.

The present study aimed to identify 281 *Brucella* isolates by conventional biotyping and molecular methods, verify the capability and usefulness of Bruce-ladder multiplex PCR for characterizing these isolates, and compare both typing methods. The study's second aim was to monitor prevalent biotypes, atypical *Brucella* strains, and vaccine strains in the field; therefore, it was expected to generate valuable epidemiological data, especially for the brucellosis control programs

Materials and Methods

Reference and test strains: Reference *Brucella* strains (*B.abortus* 544, *B.melitensis* 16 M, *B.suis* 1330, *B.canis* RM6/66, *B.ovis* 63/290, *B.neotomae* K33, *B.abortus* S19, *B.melitensis* Rev.1, *B.abortus* RB51) were from the culture collection kept at Pendik Veterinary Control Institute, Istanbul. *B.pinnipedialis*, *B.ceti*, *B.microti* and *B.inopinata* were kindly obtained by Animal Health and Veterinary Laboratory Agency (AHVLA), Weybridge, UK. A total of 281 test strains were the isolates submitted to National Brucella Reference Laboratory for species and biotype determination in three years period from 2009 to 2011.

Classical biotyping procedures: *Brucella* cultures were examined by classical biotyping procedures for the identification at species and biotype level (2, 19). Tryptic soy agar (L007516, BD) supplemented with heat-inactivated bovine serum (Biochrom, S0115, Germany) (5%, v/v) (TSA) was employed as the basal medium for all culture work. Inoculated plates were incubated at 37°C in normal atmospheric conditions and with the addition of 10% CO₂ for 4-5 days. Submitted cultures were identified in a 3-stage procedure.

Stage 1. Cultures were examined for purity and colonial morphology. For this, cultures should be in a smooth phase for typing (2, 19). For differentiation smooth and rough isolates, they were checked for colonial morphology by stereomicroscope (Olympus, VZM 294769) and for agglutination with neutral acriflavin (0.1%, w/v) (Sigma A8126). Any possible agglutination rendered any given strain untypeable.

Stage 2. For species determination, the following tests were performed on all isolates; serum requirement for growth, oxidase and urease production and lysis with Tbilisi phage at routine test dilution (RTD) and 10⁴ x RTD and R/C phage at RTD.

Stage 3. For biotyping, production of H₂S, CO₂ requirement for growth, growth in the medium containing thionine (T3387, Sigma) (20 mg/ml), basic fuchsin (115937, Merck) (20 mg/ml), and safranin O (S2255, Sigma) (100 mg/ml) dyes, agglutination with A and M monospecific antisera and R antiserum were investigated. To distinguish between field strains and vaccine strains, growth on medium containing penicillin (13750, Fluka) (5IU/ml), streptomycin (S6501, Sigma) (2.5 mg/ml), thionine blue (R310360, Sigma) (2 mg/ml) and erythritol (E7500 Sigma) (1 mg/ml) were tested.

Molecular typing of *Brucella* species by multiplex PCR (Bruce-ladder): This assay was undertaken using a published protocol (20). For extracting genomic DNA, a loopful of bacterial culture was taken from the petri plate and resuspended in 200 µl sterile distilled water. This was mixed and incubated at 99°C for 10 minutes and centrifuged at 12 000 g for 20 seconds. The supernatant was used as DNA template for Bruce-ladder. The assay was carried out in a 25µl reaction mixture containing 2× Qiagen Multiplex Master Mix (Qiagen, Germany), 2µM of each primer in cocktail of nine primer sets and 1µl template DNA. Amplifications were initiated by denaturing the sample for 15 min at 95°C was followed by template denaturation at 94°C 30 s, primer annealing at 58°C for 90 s and primer extension at 72°C for 180 s for a total of 25 cycles. After the last cycle, samples were incubated for an additional 10 min at 72°C (Palm Cycler, C61-96). Amplification products were separated by electrophoresis on 1.5% agarose gels.

Results

Results of conventional biotyping: Table 1 and Table 2 show the distribution of *Brucella* biotypes based on the animal species from which they were isolated. A total of 281 *Brucella* field isolates were processed. Of the 125 isolates from sheep and goats, 98 isolates were found to be *B.melitensis* biotype 3, 20 were identified as *B.melitensis* biotype 1 and only one isolate confirmed as *B.melitensis* biotype 2. In addition to these, 3 isolations in sheep and goats were found to be *B.abortus* biotype 3. Apart from field strains, 3 vaccine strains (2 isolations of *B.melitensis* Rev.1 in sheep and a single isolation of *B.abortus* S19 in a goat) were also identified by conventional biotyping.

Regarding to cattle isolates, 147 out of 156 isolates were found to be *B.abortus* biotype 3, whilst the remainder (9/156) were identified as *B.melitensis* strains. No vaccine strains was identified in any of the cattle isolations using conventional biotyping. Deviations from classical biotype profiles in the characterized isolates included sensitivity to penicillin and it was determined 10 *B.abortus* and 6 *B.melitensis* field isolates. Single isolation of *B.abortus* biotype 3 showed basic fuchsin sensitivity.

Table 1. Distribution of *Brucella* species and biotypes isolated from sheep and goats.

Biotype	Number of isolates	Percentage of biotype
<i>B.melitensis</i> biotype 3	98	78.4%
<i>B.melitensis</i> biotype 1	20	16%
<i>B.melitensis</i> biotype 2	1	0.8%
<i>B.melitensis</i> Rev.1	2	1.6%
<i>B.abortus</i> biotype 3	3	2.4%
<i>B.abortus</i> S19	1	0.8%
Total	125	

Table 2. Distribution of *Brucella* species and biotypes isolated from cattle.

Biotype	Number of isolates	Percentage of biotype
<i>B.abortus</i> biotype 3	147	94.2%
<i>B.melitensis</i> biotype 1	2	1.3%
<i>B.melitensis</i> biotype 3	7	4.5%
Total	156	100%

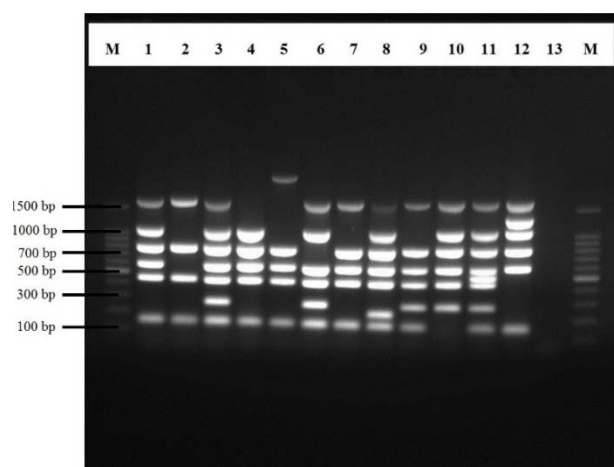


Figure 1. Bruce ladder PCR for reference *Brucella* strains. Lane M: 1500 bp ladder, Lane 1: *B.melitensis*, Lane 2: *B.abortus* S19, Lane 3: *B.suis*, Lane 4: *B.ovis*, Lane 5: *B.abortus* RB51, Lane 6: *B.canis*, Lane 7: *B.abortus*, Lane 8: *B.melitensis* Rev.1, Lane 9: *B.inopinata*, Lane 10: *B.neotomae*, Lane 11: *B.microti*, Lane 12: *B.ceti*, Lane 13: Negative control.

Results of Bruce-ladder multiplex PCR: Using the Bruce ladder protocol described by Mayer Scholl et al. (16), reference strains of all *Brucella* species that are currently known and three vaccine strains, namely *B.melitensis* Rev.1, *B.abortus* RB51 and *B.abortus* S19 displayed correct band profiles described for this test (Figure 1) Regarding to test strains, it was found that there was some degree of "host specificity" with 95% of isolates (119/125) from sheep and goats being *B.melitensis* field strains whilst 94% of isolates (147/156) from cattle were

found to be *B.abortus* field strains. However, there was a very small degree of "spill over" from "host species" with 2% (3/125) of sheep and goat isolates was identified as *B.abortus* field strains and 6% (9/156) of cattle isolates was identified as *B.melitensis*. To this end, whilst no isolations from cattle were found to be of vaccine origin, 2 isolates from sheep and goats were found to be *B.melitensis* Rev.1 and single isolation from a goat was found to be *B.abortus* S19 (Figure 2).

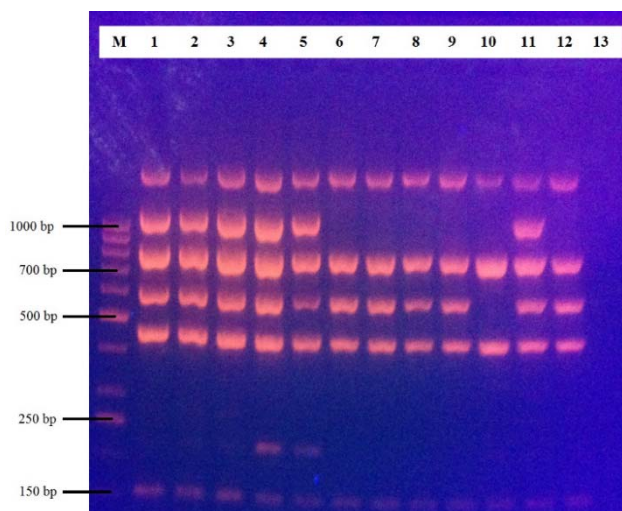


Figure 2. Bruce ladder PCR for field *Brucella* strains. Lane M: 1000 bp ladder, Lane 1-3: *B.melitensis* field isolates, Lane 4 and 5: *B.melitensis* Rev.1, Lane 6-9: *B.abortus* field isolates, Lane 10: *B.abortus* S19, Lane 11: Reference *B.melitensis*, Lane 12: Reference *B.abortus*, Lane 13: Negative control.

Discussion and Conclusion

A total of 281 *Brucella* field isolates were identified using conventional and molecular typing methods. *B.melitensis* biotype 3 is the dominant causative agent for brucellosis in sheep and goats, although other biotypes exist. However, in the case of bovine brucellosis caused by *B.abortus* biotype 3 overwhelmingly predominates in cattle. These observations are in keeping with the data from previous studies in Turkey (6, 11, 12, 16, 18, 26).

Of 125 *Brucella* isolates from sheep, 2 were identified as *B.melitensis* Rev.1 vaccine strain. This strain is known to often cause abortion and is excreted in milk when animals are vaccinated during pregnancy (4). The isolation of vaccine strains further emphasises the point that adult animals be vaccinated when they are not pregnant or during the lambing season (4, 19).

Surprisingly, one isolate from goats was identified as *B.abortus* S19 vaccine strain. This was very unusual when considering the host and the strain in question. We were informed that this goat had been reared on a goat farm where there were no other animals nearby. So, it was assumed that this animal might have been vaccinated with

a full dose of *B.abortus* S19 accidentally when she was pregnant.

The most field isolations tested exhibited typical phenotypic characteristics in keeping with conventional biotyping. Nevertheless, in the course of this study, some atypical variants were identified. All biotypes of *B.melitensis* except vaccine strains are resistant to penicillin (2, 8). However, of the 128 *B.melitensis* field isolations from sheep and goats, 6 penicillin-sensitive non-vaccine strains were identified. In the case of *B.abortus* isolations tested from all livestock, 10 were found to be penicillin sensitive non vaccine strains. In addition, one isolate of *B.abortus* biotype 3 showed sensitivity to basic fuchsin dye. Several authors have reported atypical *Brucella* isolates that do not fit into the conventional *Brucella* biotyping scheme (3, 9, 13, 25). Occasional isolates exhibit atypical characteristics that can be useful in epidemiological studies. It was thought that penicillin susceptibility might show the source of infection and could help trace back the infection (25).

In this study, nine isolates from cattle were identified as *B.melitensis*, a species more commonly found in sheep and goats. Although, there have been several reports of isolation of *B.melitensis* from cattle from different parts of the world (1, 17). It is quite reasonable to think that wherever brucellosis is enzootic in sheep and goats, the cattle living in the neighborhood have likely to acquire the infection from these species. Three of the sheep isolates were shown to be *B.abortus* biotype 3 (2.4%). Although infection with *B.abortus* is rare in sheep, there have been several reports of sheep abortion caused by *B.abortus* in various countries (21, 24). It is probable that the sheep and goats acquired *B.abortus* from the cattle on the farm, possibly from using a field that had been infected by the cattle. It has often been assumed that each *Brucella* species is known to have a definite host preference. However, inappropriate management conditions like different species of animals being kept together may allow the disease to be transferred out with traditionally accepted host species. Because of interaction between agent infectivity and host immunity and also structure of animal populations, *B.melitensis* infection in cattle may occur more frequently than *B.abortus* infection in sheep and goats. These issues should be taken into consideration when planning effective disease control programs. Animal movements also should be controlled by appropriate regulations and legislation.

Molecular typing of all the reference strains showed the correct amplicon sizes described for Bruce-ladder multiplex PCR (Figure 1). There was concordance in the results generated by both classical biotyping and Bruce-ladder in regards to species and vaccine identities. Conventional biotyping is time-consuming, requires a

high level of bacteriological expertise, safe laboratory infrastructure for both workers and environment and also hazardous to personnel who work with these agents. In addition, whilst there are selective media, such as Farrell's that limit the growth of contaminating agents (19), it can be challenging to isolate pure *Brucella* cultures from field material. These issues can be overcome by using PCR-based molecular typing tools (5, 15, 16, 20). Molecular testing does not require viable material for testing which allows for samples to be inactivated prior to DNA extraction. Furthermore, molecular tests such as Bruce-ladder have been shown to specifically work on *Brucella* spp. DNA and so would not be adversely affected by non-target DNA in contaminating agents. Further, smooth *Brucella* strains that show transition to a rough state cannot be typed conventionally because of rough lipopolysaccharides on its cell wall that render them incapable of to be agglutinated by A and M mono-specific sera and being lysed by smooth *Brucella* phages (2, 8).

Although conventional biotyping has these drawbacks, there is currently no molecular typing test capable of differentiating biotypes of *B.abortus*, *B.melitensis*, and *B.suis* (19), which is essential to understanding strain diversity within a population. In addition, it is crucial to monitor atypical strains based on phenotypical characteristics (3, 9).

In spite of the diagnostic sensitivity and specificity decreased in molecular tests when used directly from tissues to diagnose brucellosis (22), the diagnostic success of molecular tests from direct bacterial cultures is quite impressive (20, 22). Our findings offer that molecular typing is a practical test that detects all currently known *Brucella* species and can be used conveniently in diagnostic laboratories, especially where a quick diagnosis is required. Conventional and molecular typing tests have their own advantages, so it would be efficient to use both together, especially in reference laboratories and centers. Present study results explicitly demonstrated that monitoring *Brucella* isolates at regular intervals serves as a powerful epidemiological tool allowing us to know which *Brucella* species and biotypes are prevalent and the presence of any atypical *Brucella* and vaccine strains in the field. Such studies are critical in the control and accurate diagnosis of brucellosis.

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Ethical Statement

Ethical approval was not required in this study.

Conflict of interest

The authors declared that there is no conflict of interest.

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